The Role of Id Proteins in the Development and Function of T and B Lymphocytes

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

E and Id proteins are members of the basic helix-loop-helix (bHLH) transcription regulator family. These proteins control a broad range of lymphocyte biology, from the development of multiple lineages to execution of their effector functions. With the development of new experiment models, novel functions of E and Id proteins continued to be discovered. In this thesis, I focused my study on the role of Id2 in γδ T cells and $CD4^+$ αβ T cells, as well as the role of Id3 in B cells.

Id proteins have been shown to control γδ T cell development. Id3 knockout mice demonstrate a dramatic expansion of innate-like $V\gamma1.1^+$ $V\delta6.3^+$ γδ T cells in the neonatal stage, suggesting that Id3 is an inhibitor of their development. Interestingly, Id3 knockout mice with a B6/129 mix background have much less expansion of the $V\gamma1.1^+$ $V\delta6.3^+$ γδ T cells compared to mice with pure B6 background. Genetic studies showed that this difference is strongly influences by a chromosome region very close to the *Id2* locus. Using the $Id2^{f/f}$ $CD4Cre^+$ mice, I found that Id2 is also an inhibitor of γδ T cell development. Deletion of Id2 alone is sufficient to enhance the maturation of these cells in the thymus and induce a moderate expansion of γδ T cells in the periphery. This study demonstrated the delicate balance of transcription control in cells of the immune system.

The $Id2^{f/f}$ $CD4Cre^{+}$ mice also enabled me to study the role of Id2 in peripheral $CD4^{+}$ $\alpha\beta$ T cell functions, which was difficult in the past because Id2 knockout mice lack lymph node development. I found that CD4 T cells in these mice have a profound defect in mounting immune responses, demonstrated by a complete resistance to induction of experimental autoimmune encephalomyelitis (EAE). I found that Id2-deficient CD4 T cells fail to infiltrate the central nervous system, and the effector CD4 T cell population is smaller compared to that in control mice. Id2 is important for the survival and proliferation of effector CD4 T cells, and this phenotype was correlated with an increased expression of Bim and SOCS3. This study revealed a novel role of Id2 in the functioning of $CD4^{+}$ $\alpha\beta$ T cells.

Switching my focus to B cells, recent next generation sequencing of human Burkitt's lymphoma samples revealed that a significant proportion of them have mutations of Id3. This finding suggests that Id3 may be a tumor suppressor gene in the lymphoid system. Utilizing various Id3 knockout and conditional knockout mouse models, I showed that Id3 deficiency can accelerate lymphoid tumor genesis driven by the over-expression of oncogene c-Myc. This work may lead to development of a more realistic mouse model of human Burkitt's lymphoma, allowing more mechanistic studies and perhaps preclinical tests of new therapies.

Dedication

To Yu-Hui.

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Acknowledgements

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Lastly, I want to thank my wife, Yu-Hui Chan. Thank you for supporting me to pursue my dream. Now it's your turn.

1. Overview

The immune system is unique among the systems of the multi-cellular organism. Unlike other systems, which may be near mature at birth, the immune system continues to develop and adapt after the birth of the animal. However, all cells of the immune system develop and differentiate from the same hematopoietic stem cell (HSC) throughout the animal's life span. The differentiation process, the formation of individual progeny clones, the execution of the effector functions, and the formation of immune memory are all tightly regulated, yet the system can still rapidly change to respond to environmental challenges and protect the organism. A delicate genetic network controls all of these processes, with surprisingly few but versatile genes capable of influencing both the development and response of immune cells. The E protein and Id protein transcription regulators are prime examples of such multi-functional regulators of the immune system (Kee, 2009). They are multifunctional genetic factors that play critical roles in the immune system starting from lymphocyte development as early as before birth to the generation of immune memories that may last decades. Research on these molecules revealed to us the complex architecture of the immune system, and this knowledge can instruct us on how to carefully develop therapies to manipulate the immune system for a variety of human diseases.

1.1 Introduction to E proteins and Id proteins

E proteins and Id proteins are basic helix-loop-helix transcription regulators that have a broad impact on the immune system (Murre, 2005). In mammalian immune systems, there are three major E proteins (E2A, HEB, E2-2) and two major Id proteins (Id2, Id3) expressed by a variety of cell types. The E proteins can homo-dimerize or hetero-dimerize with their helix-loop-helix domains, and the dimers bind to DNA sequences containing the E-box (CANNTG) with their basic DNA binding domains. The sequence simplicity of E-box implies that a large number of genes may contain such sequences and be subject to E protein control. The binding of E protein can recruit transcription co-activator or co-repressor to the target gene and promote or repress its expression. On the other hand, the Id proteins also contain helix-loop-helix domains but not basic DNA binding domains. Therefore, Id proteins can sequester E proteins and prevent them from binding to target DNA, essentially functioning as E protein inhibitors.

The E protein – Id protein interaction network is very adaptable during the development of immune cells as well as the development of the entire organism. The activity of E proteins is regulated by the expression level of individual E proteins and the inhibition from Id proteins. Different cell types have different levels of E protein activity and target gene preferences. Therefore, it is not surprising that this E – Id axis is involved in many different events in the immune system, including hematopoietic stem cell homeostasis and differentiation, the development of T and B lymphocyte, innate

lymphoid cell and plasmacytoid dendritic cell, the formation of lymph node, peripheral T cell response, and hematopoietic cancer formation (Kee, 2009; Spits and Di Santo, 2011; Yang et al., 2011). Novel functions of the E – Id axis are continuing to be discovered. Below, the discussion will be focused on what is currently known about the role of E protein and Id protein in T lymphocyte development / function and lymphoid tumor formation, and how novel materials and methods may further advance our understanding of this transcription control system, especially about Id2 and Id3.

1.2 E and Id in the Transcription Control of T Cell Development and Function

T lymphocytes develop in the thymus and migrate to the peripheral lymphoid organs, where they interact with antigen presenting cells, become activated, and carry out their effector functions, such as killing infected cells or producing cytokines. E and Id proteins have been shown to be involved in many stages of T lymphocyte development and function, such as during the commitment to T cell lineage, αβ/γδ lineage choice, CD4/CD8 lineage choice, as well as during peripheral T cell homeostasis and function.

1.2.1 Commitment to the T cell lineage

In the bone marrow, the hematopoietic stem cells produce all the cell lineages of the hematopoietic system. Early in the differentiation process, the stem cells give rise to two different progenitors: one with potential to produce myeloid cells, such as neutrophils and eosinophils, and one with potential to produce lymphoid cells, such as

B cells and T cells. The lymphoid progenitor cells further differentiate into T cell progenitors and B cell progenitors, and the T cell progenitors leave the bone marrow and migrate to the thymus, where they continue their differentiation into mature T lymphocytes (Kindt et al., 2007).

E proteins start to influence the development of T cells from the very beginning. The E2A-deficient HSCs produced reduced numbers of lymphoid-primed multipotent progenitor cells (LMPPs), and these cells have reduced potential to produce cells of the lymphoid lineage but instead tend to differentiate into granulocytes and macrophages (Dias et al., 2008a). E2A seems to cooperate with transcription factor PU.1 to generate a "lymphoid priming" effect in these progenitor cells and increase the expression of multiple genes unique to the lymphoid lineage, thus promoting the differentiation toward that lineage (Dias et al., 2008b).

Once committed to the lymphoid lineage, E2A continue to play a crucial role in driving B cell development through activation of a cascade of B cell-specific transcription factors, including EBF1 and Pax5 (Hagman and Lukin, 2006). It has been proposed that cells which receive Notch signaling will instead adopt a T cell fate, at least partially through the antagonizing of E2A regulation on target genes by the Notch pathway (Tanigaki and Honjo, 2007). Interestingly, E2A itself can also up-regulate Notch expression, making the cells sensitive to Notch ligand signaling (Ikawa et al., 2006). In the absence of E2A, B cells fail to develop, but T cell numbers are also reduced,

indicating that E2A does not simply promote or repress the choice of differentiation into T cells, but it probably is involved in a more complicated network.

The lymphoid progenitor that picks up the propensity to differentiate into the T cell lineage will migrate out of the bone marrow and into the thymus. In the thymus, the progenitors further differentiate into either cells that give rise to innate lymphoid cells (including NK cells, lymphoid tissue inducer cells, etc) or cells that give rise to T cells. Id2 is crucial at this step; its presence suppresses the E protein activity and allows the development of all innate lymphoid cells (Mjosberg et al., 2012). For those cells that do not up-regulate Id2 at this stage, they proceed down the path to become one of two kinds of T lymphocytes: αβ or γδ T cells.

1.2.2 αβ/γδ lineage choice and γδ T cell development

The early T cell progenitors do not express T cell markers such as CD4 or CD8, thus called double negative (DN) cells. Most DN thymocytes will eventually rearrange one of two families of T cell receptor genes: the αβ or the γδ receptor, and the fate of the cells will be determined by this event. Those which successfully rearrange the αβ receptor will become αβ T cells, while rearrangement of the γδ receptor leads to the γδ T cell fate. During fetal development and the neonatal life, most T cells adopt the γδ T cell fate, but as the animal matures, αβ T cells quickly become dominant, account for the majority of the thymic output in adults (Havran and Allison, 1988). How this transition

occurs, and what determines the αβ/γδ lineage choice, have long been fascinating questions to immunologists.

Two different hypotheses have been proposed to explain how thymic progenitor cells make the lineage choice decision. The first is the "pre-commitment" model, stating that some progenitor cells express key transcription factors for the γδ lineage (such as SOX13) in a stochastic manner, and these cells are "committed" to become γδ T cells (Melichar et al., 2007). The rearranged T cell receptor type must match the pre-commitment of the cell for it to continue its development. The other hypothesis emphasis the role of the T cell receptor produced in the rearrangement process. The "instructive model" states that the T cell receptor has a decisive role of instructing the fate of the cell, and this involves the E – Id axis. Proponents of this hypothesis observed that αβ and γδ T cell receptors possess certain different qualities, and these differences can determine the subsequent development of the cells. For example, the γδ T cell receptors have been shown to be generally capable of sending a stronger intracellular signal into the cell when compared with the TCRβ-pTα T cell receptors in the DN cells (Hayes et al., 2003). Such signal involves activation of the MAPK pathway, which can lead to up-regulation of one of the Id proteins, Id3, and inhibit the activity of E proteins (Lauritsen et al., 2009). The resulting difference in E protein-controlled transcription network may promote the cell to adopt a program specific to the γδ T cells, thus diverting the cells from the αβ T cell fate.

Once the cell picks the γδ T cell fate, it proceeds toward a different differentiation pathway from the rest of the αβ-destined T cells. The cell ceases to rearrange the β T cell receptor locus and does not rearrange the α locus. Instead, it starts to differentiate into effector-like cells. This is different from the αβ T cells, as the majority of αβ T cells retain a naïve phenotype in the thymus. The γδ T cells may differentiate into either IFNγ producing or IL-17 producing cells. It has been shown that if a developing γδ T cell encounters its cognate antigen in the thymus, it becomes an IFNγ producer, while those that do not encounter an antigen become IL-17 producers (Jensen et al., 2008). Unlike the αβ T cells, among which the ones that react with self antigens strongly will be negatively selected and deleted, autoreactive γδ T cells actually survive and migrate to the periphery. It is believed that these cells function as innate-like sensors of danger signals and respond rapidly to the release of self molecules in stressed tissue by producing inflammatory cytokines including IFNγ (Vantourout and Hayday, 2013). On the other hand, the IL-17 producing cells do not receive TCR signaling from antigen in the thymus, and these cells also seem to be rapidly responding to non-TCR signaling, such as inflammatory cytokine signaling, in the periphery (Cai et al., 2011). They also respond to such stimulations promptly by pumping out more IL-17, contributing to tissue inflammation. There are also γδ T cells that respond to foreign antigen stimulation in a genuinely adaptive fashion; for example, phycoerythrin (PE) specific γδ T cells have been identified in both mouse and human, and these cells can

respond to PE stimulation through their γδ TCR without the aid of MHC presentation, become activate, expand and produce effector cytokines (Zeng et al., 2012).

Another important distinction between αβ and γδ T cells is that γδ T cells produced at different developmental stages utilize special semi-invariant TCR V segments. In human, the first wave of γδ T cells produced during fetal development utilizes the Vδ2 segment and circulate in the blood, while the γδ T cells produced later during neonatal life utilize the Vδ1 segment and populate the mucosal surfaces and internal organs (Krangel et al., 1990; Pang et al., 2012). In mice, multiple waves of γδ T cells develop throughout embryonic development and early life, utilizing Vγ5, 6, 4 and 1 segments sequentially (Carding and Egan, 2002). These different waves of cells migrate to specific target organs and reside there throughout the life span of the animal, largely maintained by homeostatic proliferation, with little further thymic output supply. How these TCR V segments are used in such a sequential and orderly manner, and how their usage eventually largely stops and be replaced by αβ T cells are interesting questions. For a certain subset, the skin-directed dendritic epithelial T cells (DETC), which utilize the Vγ5 segment (alternatively named Vγ3), a specific gene, *skint1*, has been identified to be essential for their development ((Boyden et al., 2008)). One may speculate that controlled expression of other genes may similarly dictate the timely development or selection of γδ T cells utilizing other V segments.

Among these specific waves of γδ T cells, not all of them require a high level of Id3 to develop. In the absence of Id3, the development of certain subsets of γδ T cells actually is greatly enhanced. The rearrangement of γδ TCR genes, especially those involving the Vγ1.1, is enhanced in Id3 knockout mice (Ueda-Hayakawa et al., 2009), and the total number of γδ T cells expressing the Vγ1.1 and Vδ6.3 T cell receptor gene segments increases dramatically (Alonzo et al., 2010; Ueda-Hayakawa et al., 2009). How these cells are differently regulated from other γδ T cells, and how the E – Id axis influence their development will be the focus of Chapter 2.

1.2.3 CD4/CD8 lineage choice and thymic selection

For the majority of thymocytes that adopt the αβ T cell fate, they proceed from the DN stage into DP (double positive) stage, when they express both CD4 and CD8 on their surface. The cells rearranged their TCR β chain in the DN stage, and they rearrange their TCR α chain in the DP stage. In the DP stage, the cells again make a lineage choice to become either a CD4 single positive (SP) cell or a CD8 single positive cell. The SP cells are mature and ready to migrate to the periphery to carry out effector functions.

The T cell receptor again plays a significant role in this decision. Cells expressing receptors capable of interacting with class I major histocompatibility (MHC) molecules go on to become CD8 SPs, while those with receptors binding class II MHCs become CD4 SPs. The TCR-MHC interaction also sustains the survival of the T cells, so the process is termed "positive selection". Those that fail to interact with either class of

MHCs die through apoptosis, due to a lack of survival signal (death by neglect), and do not proceed to the next stage. However, how does a DP cell, which express both CD4 and CD8, tell the difference between a TCR signal that is coming from class I or class II MHC molecules? Similar to the choice between αβ and γδ lineages, both instructive model and stochastic model have been proposed. The stochastic model suggests that DP cells will randomly down-regulate either CD4 or CD8; because CD4 and CD8 are important for stabilizing the interaction between MHC and TCR, a T cell with a TCR binding to class II MHC will only keep receiving signals and survive if it down-regulates CD8 and keeps CD4, and the opposite is true for T cells recognizing class I MHC (Chan et al., 1994). The instructive model (Singer et al., 2008), on the other hand, suggests that there are qualitative difference between TCRs recognizing class I and class II MHCs. Dr. Alfred Singer's lab observed that the downstream signal is typically stronger from an interaction between CD4, TCRs and class II MHC molecules, and they found that DP cells go through an intermediate stage when they down-regulate CD8 and become $CD8^{low}CD4^{+}$ cells. At this stage, T cells that express TCRs recognizing class II MHC molecules maintain their interaction with the help of CD4, while those that express TCRs recognizing class I MHC molecules lose their TCR signal, at the same time gaining IL-7 receptor expression and receiving IL-7 signal (Brugnera et al., 2000). These differences lead to either maintenance of CD4 expression, or a switch to CD8 expression, generating CD8 SP and CD4 SP cells, respectively.

The CD4/CD8 lineage choices, as well as the DP survival check point, again are both involving the E – Id axis. When the DP thymocytes successfully rearrange the TCRα gene and express the $\alpha\beta$ TCR on the surface, and the receptors interact with one of the MHC molecules, again a signal will be sent into the cell. This signal again can up-regulate Id3 expression and inhibit the activity of E proteins, thus permitting the cells to pass through the "check point" and change the transcription program to one of the SP cells (Engel et al., 2001). If the two major E proteins, E2A and HEB, are both deleted in the DP thymocytes, the cells may proceed to become SP cells even without a successfully rearranged T cell receptor, partially eliminating the requirement of positive selection (Jones and Zhuang, 2007).

However, in the aforementioned experiment, all the TCR-negative "SP" cells express CD8, and no CD4 SP cells are formed. This indicates that the E – Id axis also influences the CD4/CD8 lineage choice. The opposite mouse model, with deletion of both Id3 and Id2 in DP cells, results in no TCR-negative SP cells, and all SP cells thus formed are CD4 SP (Jones-Mason et al., 2012). This indicates that higher E protein activity is important for the CD4 SP fate, while the lack of it is compatible with the CD8 SP fate. The effect may be mediated by the E protein suppression of IL-7 receptor α expression. It is mentioned above that CD8 SP differentiation requires IL-7 signaling. When Id2 and Id3 are deleted, E protein activity becomes too high, and IL-7 receptor α

expression decrease; this situation can be inhibitory to CD8 SP cell development (Jones-Mason et al., 2012).

Before the mature SP cells leave the thymus, a final check point they must pass is the negative selection. Cells that are strongly reactive to self antigens will be deleted, thus preventing the formation of autoimmunity. The medullary thymic epithelial cells (mTECs) express a special transcription factor AIRE that can induce nonspecific expression of tissue antigens on their surface, therefore enabling the screening of T cells reactive against different tissues (Metzger and Anderson, 2011). In at least one case, this negative selection also involves Id3. In the Id3 deficient male mice, T cells specific for the male antigen H-Y are not deleted and can migrate to the periphery (Rivera et al., 2000). This may be explained by Id3 mediating downstream signaling of the TCR; in the absence of Id3, the TCR signal strength is generally weakened, so cells that normally die because of strong TCR interaction with self antigen now survive. The E – Id axis is indeed important for the development of T lymphocytes from the beginning to the end.

1.2.4 Peripheral T cell homeostasis and function

The T cells that have successfully gone through all the differentiation processes and check points eventually leave the thymus and migrate into the periphery. These cells circulate throughout the body in a naïve, quiescent state, surveying the antigen presenting cells in the body constantly. If a naïve T cell encounters its cognate antigen, such as an antigen from an infectious agent, the T cell can become activated and

proliferate vigorously to form a large population of effector T cells to carry out immune defense functions, such as killing infected cells or secreting cytokines. If the infection is cleared, most effector T cells will die by apoptosis, and only a small population of cells will survive and become long-lived memory cells. If the same antigen is encountered again, the memory cells can re-activate and mount a secondary immune response much faster and stronger than the primary immune response, thus offering the host a superior level of protection.

No other mammalian tissue is regulated the same way as the lymphocytes. A large pool of diverse naïve lymphocytes are maintained for a long time with little apparent change; upon challenge, a very selective subset must burst into proliferation quickly; once the challenge is gone, the majority of the effectors must die, and memory cells must survive, sometimes for a very long period of time. This is in stark contrast to the epithelial cells, which basically are renewed consistently at a steady speed, or to the neurons, which basically don't proliferate much after birth. The lymphocytes must have an extraordinarily flexible population size control mechanism.

The overall "theme" of lymphocyte population control mechanism may be summarized as the following: "die unless instructed otherwise, plus always be ready to proliferate." Just like in the thymus, T cells that do not receive survival signals, such as when isolated in *in vitro* culture, very quickly die. The naïve T cells require constant low level stimulation to their T cell receptors (TCRs) from MHCs loaded with self peptides to

maintain their survival (Surh and Sprent, 2008). They also require IL-7, which is produced by various tissues, including fibroblastic reticular cells in the T cell zones of secondary lymphoid organs (Link et al., 2007). These signals are important for the continuous expression of anti-apoptotic proteins, such as Bcl-2 and Mcl-1, to keep the cells from dying (Khaled and Durum, 2002). If a T cell encounters its cognate antigen, the peptide-MHC interaction with the TCR is typically stronger, and second and third signals provided by co-stimulatory molecules (such as CD80 and CD86) and cytokines (such as IL-2) together can activate the survival and proliferation program in the lymphocyte. As long as these signals persist, the T cell can continue to survive and proliferate, with the exception of chronic infections, in which situation the T cell response may be dysregulated or dampened. During an immune response, multiple transcription factors capable of driving cell growth and proliferation will be turned on, such as c-Myc and AP-1 transcription factors (Hayashi and Altman, 2007; Yang and Chi, 2012). But activated cells are also highly susceptible to cell death. Activated T cells increase expression of Fas ligand, which when ligating to Fas expressed on other T cells can induce the T cells to go through apoptosis (Green et al., 2003). Yet the subset of T cells that become memory cells is different. They are less proliferative and produce less effector molecules, but they are also less susceptible to cell death. Even when the antigen is cleared and the TCR stimulation ceases, the memory T cells can continue to survive. These cells now require less of the signal from self peptide-loaded MHC molecules, but

they gain the responsiveness to IL-15, in addition to IL-7 (Surh and Sprent, 2008). They also gain a low level of turn over capability, replenishing their numbers by slow proliferation (Tough and Sprent, 1995).

The research of T cell homeostasis is currently ongoing in several directions. One of the active fields is the differentiation of effector cells versus memory cells. Which 5% of the total T cells formed in an immune response are going to become memory T cells? There are three different possible mechanisms (Amsen et al., 2013). The first theory suggests there are pre-committed populations of T cells at the very beginning of an immune response. The cells that receive different signals when they are activated are pre-destined to become either effector or memory T cells. The second theory argues against such a pre-commitment; instead, it postulates that all cells have equal potential of becoming memory, and through competition or random chance, only a small proportion of the cells receive sufficient survival signals and persist. A third theory is poised between the previous two, suggesting that in the early phase of an immune response, the majority of the cells produced become effector cells, while in the later stage, when the triggering pathogen is cleared and inflammation is subsiding, the T cells receive less vigorous stimulation and are more prone to become memory cells. Each theory has its own supporting experiment evidence. It is possible that the formation of memory is a fine-tuned phenomenon and is slightly different in different immune responses, and in some cases it is more predestined, while in others the effectors are less

terminally differentiated and retain memory potential. In fact, the memory T cells are known to be a heterogeneous population. The so called "effector memory cells" have phenotypes of effector T cells, patrol the mucosal surfaces, can produce effector molecules like cytokines rapidly, have a long life span but do not proliferate much. On the other hand, the "central memory cells" are more similar to naïve T cells, reside in the secondary lymphoid organs, do not produce effector molecules, live long and can proliferate and differentiate into new effector cells. The effector memory cells are considered to provide more direct protection for the host, while the central memory cells are crucial in forming the secondary immune response. Recently, a population of "memory stem cells" was identified to be the progenitor of both effector memory and central memory cells (Gattinoni et al., 2009). This rare population of cells has a gene expression profile that is similar to quiescent naïve T cells and can confer even better protection to a recipient of the cells than the other two types of memory cells in a transfer experiment. How all these populations differentiate and are maintained is an active area of research, for the knowledge is potentially useful for the design of vaccine strategies.

The differentiation and maintenance of CD4 T cells versus CD8 T cells appear to follow different mechanisms. A very significant difference is that CD4 memory T cells appear to decline over time (Homann et al., 2001). This may be caused by their relatively low expression of CD122, which is the receptor of IL-2 and IL-15; therefore, they cannot

compete with the CD122^{hi} memory CD8 T cells and NK cells for cytokine support for survival (Purton et al., 2007). Another possibility is they are constantly being replaced by CD4 T cells that have TCRs with higher affinity for either the foreign antigen or self antigen, so the observed memory population does not persist (Williams et al., 2008). While the formation of CD8 memory T cells requires help from CD4 T cells, it has been shown that the formation of CD4 memory T cells may require help from B cells (Pepper and Jenkins, 2011). Naïve CD4 T cells also require a longer period of stimulation than CD8 T cells to undergo efficient differentiation into memory cells (Williams et al., 2008). These factors may all contribute to the difference between CD4 and CD8 memory T cell maintenance. Nevertheless, both populations require IL-7 and IL-15 to survive and turnover.

E proteins and Id proteins also have roles in the population size control of peripheral T lymphocytes, including all the naïve, effector and memory populations. Unlike double deletion of Id2 and Id3, loss of Id3 alone allows development of CD4 and CD8 SP cells, but the deletion results in a loss of the naïve phenotype, i.e., the mature T cells can spontaneously become activated (Miyazaki et al., 2011). Without Id3, the unrestrained T cells can adopt an effector-memory phenotype right in the thymus and even induce B cell follicle formation (Miyazaki et al., 2011). Id3-deficient CD4 T cells also have reduced potential to develop into regulatory T cells but higher potential to become pro-inflammatory Th17 cells (Maruyama et al., 2011). Combined with a thymic defect of

negative selection in Id3 knockout mice (Rivera et al., 2000), these phenomena of enhanced activation and inflammation may contribute to the spontaneous development of Sjogren syndrome, an autoimmune destruction of exocrine gland, in the Id3-deficient mice (Li et al., 2004). However, Id3 is also important for the formation of memory T cells. Two reports showed that Id3 supports the survival of effector and memory CD8 T cells following virus and bacteria infections (Ji et al., 2011; Yang et al., 2011).

Single deletion of Id2 results in a milder phenotype in mature T lymphocytes in the steady state, with largely normal numbers of CD4 and CD8 T cells in the periphery. However, the importance of Id2 is demonstrated when the cells are activated. Id2-deficient CD8 T cells form a smaller pool of effector cells in a *Listeria monocytogenes* infection model, and these cells form a smaller number of memory cells, especially decimating the effector memory cells (Cannarile et al., 2006). How Id2 functions in the CD4 T cell immune responses is less well studied, and this will be the focus of discussion in Chapter 3.

1.3 E and Id in the Development of Lymphoid Tumors

Considering the complexity of regulations in the development of the lymphoid system, and the explosive proliferation potential of the lymphocytes, it is not surprising that many different kinds of human and mouse malignancies arise from the lymphoid system. Cancer can develop from cells of almost any stage of development. The surface

markers they express can indicate their origin, serve as diagnostic standards, and sometimes even hint at their behavior and potential clinical prognosis.

Human malignancies of lymphoid cells can be largely divided into leukemias (those involving bone marrow and blood) and lymphomas (those forming solid tumors), although sometimes the lymphomas can also progress into leukemias. Most common malignancies belong to either the T cell type or the B cell type, with B cells accounting for the majority of cases (**Fig 1** and **Fig 2**) (Longo, 2011). The malignancies arising from the early progenitor cells typically present as acute leukemias, flooding the patient's bone marrow and blood with blasts, while those arising from mature, peripheral populations often behave more indolently, sometimes remaining asymptomatic for decades. However, this distinction is by no means absolute, and the apparent stage of differentiation of the cancer cells does not directly indicate the stage at which the cells acquired their genetic changes and became transformed.

A distinctive feature of the lymphoid malignancy is that many of them have recurrent genetic abnormalities, including chromosome translocations, that can be causally linked to the malignant transformation process (Longo, 2011). Such clear mechanisms of transformation are unique and not frequently seen in tumors of other organ systems. Many such events involve translocation of chromosomes bring proto-oncogenes to the immunoglobulin genes (in B cells) or the T cell antigen receptor genes

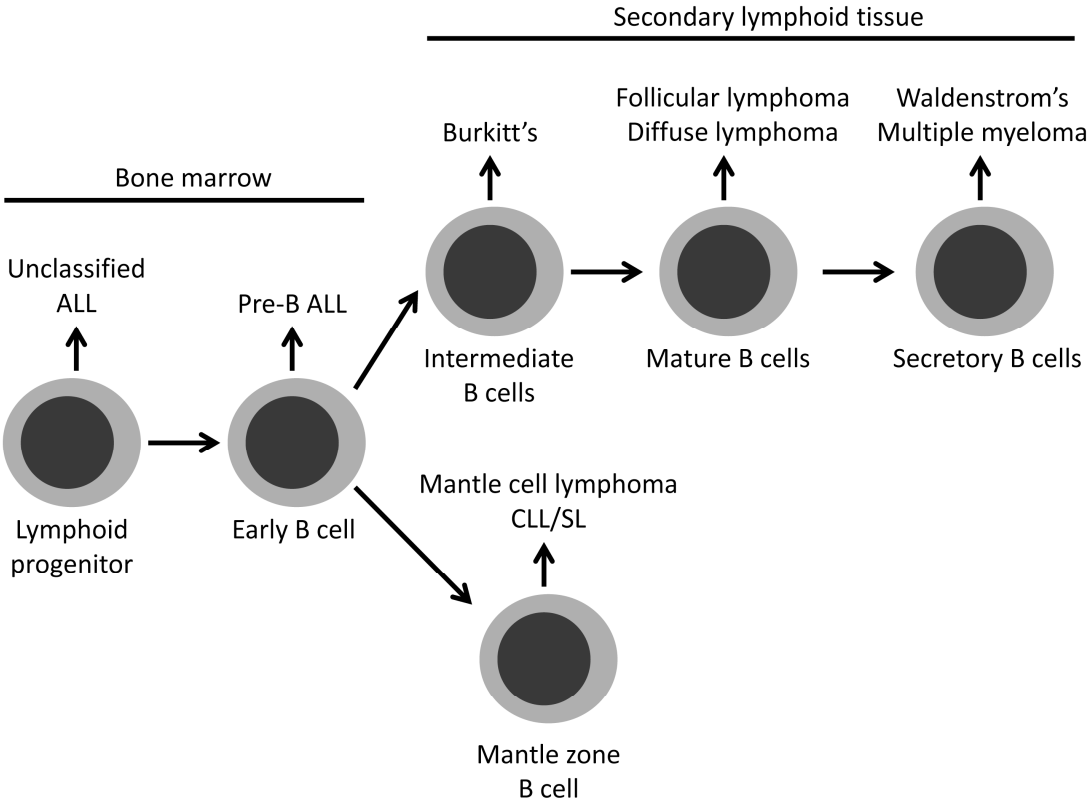

Figure 1: Human B cell lymphomas and their approximate relationship to normal B cell differentiation

ALL, acute lymphoid leukemia; CLL, chronic lymphoid leukemia; SL, small lymphocytic lymphoma. Adapted from: Chapter 110. Malignancies of Lymphoid Cells. Harrison's Principles of Internal Medicine, 18e, 2011.

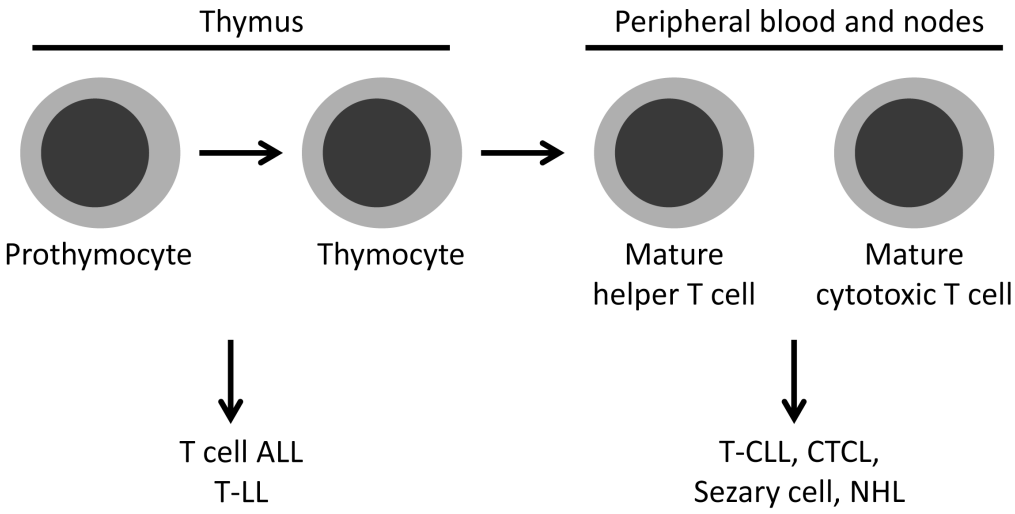

Figure 2: Human T cell lymphomas and their approximate relationship to normal T cell differentiation

ALL, acute lymphoid leukemia; T-ALL, T cell ALL; T-LL, T cell lymphoblastic lymphoma; T-CLL, T cell chronic lymphoid leukemia; CTCL, cutaneous T cell lymphoma; NHL, non-Hodgkin's lymphoma. Adapted from: Chapter 110. Malignancies of Lymphoid Cells. Harrison's Principles of Internal Medicine, 18e, 2011.

(in T cells). Rearrangement of these antigen receptor genes during lymphocyte development makes them frequent targets of chromosome translocation, and their strong expression in the individual lineages can boost the expression of proto-oncogenes translocated to their proximity. Other than translocations, gene deletion or amplification, as well as mutation, also contribute to the transformation of different types of lymphoid cancers. Some of the oncogenes control cell death or proliferation, while others are transcription factors capable of disrupting lymphocyte differentiation when inappropriately expressed. Again, E proteins and Id proteins are also involved in this process.

The peripheral CD8 T cell study mentioned above demonstrated that a proper balance between E proteins and Id proteins is not only essential for developing lymphocytes to progress through the differentiation processes, but it is also critical for controlling the cell population size at each stage. In fact, early studies of E2A knockout mice showed that these mice can develop a very aggressive T cell lymphoma (Bain et al., 1997; Yan et al., 1997). Human study also found that E2A is often lost in cancer cells from patients with Sezary syndrome, a type of peripheral T cell lymphoma (Steininger et al., 2011). However, loss of Id3 has also been shown to be oncogenic. Id3 knockout mice can develop a γδ T cell lymphoma (Li et al., 2010). In human, a significant proportion of Burkitt's lymphoma cases carry bi-allelic mutation of Id3, and restoring Id3 expression in the cancer cells resulted in cell death (Schmitz et al., 2012). These results imply that

E2A can also be an oncogene in certain situations, and Id3 becomes a tumor suppressor gene by inhibiting E2A. These conflicting results again highlight that the activity of E proteins and Id proteins need to be tightly and differentially regulated among different cell types, and an imbalance can easily result in either loss of the cell population or uncontrolled neoplasm.

E2A has also been shown to be involved in several chromosome translocation and gene fusion events in different types of lymphoma/leukemia, such as the E2A-PBX1 and the E2A-HLF fusions seen in 1 to 5% of childhood B cell precursor acute lymphoblastic leukemia (Aspland et al., 2001; LeBrun, 2003). The fusions result in the combination of the trans-activating domain of E2A and the DNA binding domain of the partner gene. These cancer cells often have deletion of the other non-translocated E2A allele. This results in loss of E2A regulation on its own target but enhanced or repressed transcription of the targets of the fusion partner. For example, E2A-PBX1 fusion can increase the expression of Wnt16, a component of the Wnt signaling pathway, while E2A-HLF can increase the expression of ABCB1, a membrane transporter that can render the cancer cells resistant to chemotherapies (LeBrun, 2003).

1.4 New Materials and Methods Enable Investigation of E and Id Function in Previously Overlooked Compartments

Although so much is known about the role of E and Id proteins, their function in several aspects of the immune system has never been probed in the past because of the

limitation of previously available materials and methods. For example, whether Id2 plays a role in peripheral CD4 T cell responses was not known because Id2 knockout mice simply have no lymph node development (Yokota et al., 1999), which is important for CD4 T cell response to occur. The role of Id proteins in the development of γδ T cell development after they commit to the γδ lineage was not well studied, either; while Id3 single knockout mice have a tremendous expansion of $V\gamma1.1^{+}$ $V\delta6.3^{+}$ cells, indicating an inhibitory role of Id3 in the development of this subset of γδ T cells, whether Id2 plays a synergistic or compensatory role in their development is not known, since Id2 and Id3 double knockout mice are not viable. And attempts to model human Burkitt's lymphoma through c-Myc over-expression and Id3 knockout in mice was complicated by these mice's preferential development of T cell, not B cell, lymphoma, as will be demonstrated in Chapter 4. We need to turn to newer conditional, tissue-specific knockout models of Id2 and Id3, as well as reporter strains, to address these questions.

In this thesis, I will discuss my work considering the following questions: first, does Id2 collaborate with Id3 in the control of γδ T cell development? Second, does Id2 influence peripheral CD4 T cell responses? Third, does Id3 collaborate with c-Myc in the formation of Burkitt's's lymphoma? These questions are explored in detail in the following three chapters.

2. Id2 is an inhibitor of the development of γδ T cells

Part of the information in this chapter is originally published in *The Journal of Immunology*. Baojun Zhang, Yen-Yu Lin, Meifang Dai, and Yuan Zhuang. 2014. Id3 and Id2 act as a dual safety mechanism in regulating the development and population size of innate-like γδ T cells. J. Immunol. Vol 192(3):1055-63. Copyright © 2014 The American Association of Immunologists, Inc.
(http://www.jimmunol.org/content/192/3/1055.abstract)

2.1 Introduction

γδ T cells are a subset of T lymphocytes generated in the thymus that function between the innate and adaptive immune system. They have features of the adaptive immune system, such as the expression of variable rearranged γδ T cell receptors, but they also have features of the innate immune system, such as the ability to respond to stimulation rapidly (Bonneville et al., 2010). They can directly lyse infected or stressed cells as well as interact with αβ T cells, B cells and dendritic cells and regulate their functions (Vantourout and Hayday, 2013). As a result, γδ T cells are involved in a broad range of immune processes, such as infection, inflammation, autoimmunity, tumor surveillance and tissue maintenance (Bonneville et al., 2010; Carding and Egan, 2002). These cells are produced in large numbers in the fetal and neonatal stages in mammals, disseminating and forming stable populations in internal organs, mucosal and body

surfaces, but their thymic production is gradually replaced by αβ T cells when the animal matures (Xiong and Raulet, 2007). The mechanism that controls the developmental switch from γδ to αβ T cell production in the thymus is not fully understood.

Among γδ T cells, the cells that express the Vγ1.1 and Vδ6.3 segments of the γδ T cell receptor belong to a unique subset. In mice, these cells are produced in large numbers in the neonatal thymus (Grigoriadou et al., 2003) and are capable of rapidly producing multiple cytokines, including IFNγ and IL-4, upon stimulation (Azuara et al., 2001). They express the transcription factor PLZF that is also found in NKT cells (Alonzo et al., 2010). Like NKT cells, they also have a significant presence in the liver (Gerber et al., 1999). The semi-invariant nature of their T cell receptor and their response pattern lead to the classification of these cells as "innate-like γδ T cells." Although their function is not clearly understood, several studies pointed out that these cells may play an important role in attenuating excessive inflammation during infection and autoimmune processes due to their unique ability among γδ T cells to produce Th2-like cytokines (as reviewed by (Carding and Egan, 2002)). The population size of $V\gamma1.1^{+}V\delta6.3^{+}$ γδ T cells varies between mouse strains; they are particularly abundant in the DBA strain (in which usually a Vδ6.4 segment is expressed) but relatively rare in the B6 strain (Azuara et al., 2001). However, in the absence of the helix-loop-helix transcription regulator, Id3,

it has been shown that Vγ1.1$^+$Vδ6.3$^+$ γδ T cells can also expand dramatically in mice with B6 genetic background (Ueda-Hayakawa et al., 2009).

As mentioned in Chapter 1, Id3 has been implicated to play both positive and negative roles in the developmental control of γδ T cells. It has been shown that in developing DN thymocytes, if a cell successfully rearranges the γδ T cell receptor genes, the surface expression of γδ T cell receptor can send a strong signal into the cell and up-regulate Id3, promoting the cell to adopt a γδ T cell fate (Lauritsen et al., 2009). However, Id3 must also play a distinct inhibitory role controlling the development of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells because this population is dramatically expanded in Id3 deficient mice. More interestingly, this expansion is limited to the neonatal window and cannot be recapitulated by transferring Id3-deficient bone marrow cells into adult wild type B6 animals (Verykokakis et al., 2010). The expansion also requires a pure B6 genetic background; in a B6/129 mix background, the expansion is variable and often greatly diminished (Ueda-Hayakawa et al., 2009). The latter phenomenon suggests that additional gene(s) specific to the B6 background is also critical in the development of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells in the absence of Id3.

This strain- and genotype-specific expansion of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells represents a unique opportunity to identify novel players in the developmental control of γδ T cells. In our lab, Baojun Zhang designed a backcross experiment between B6 and 129 Id3-deficient mice with a goal to identify the background genes in regulating the

Vγ1.1^{+}Vδ6.3^{+} γδ T cells. He found that another member of the Id protein family, Id2, was the major modifier of Id3 involved in the control of γδ T cell population size (Zhang et al., 2013). In B6/129 hybrid Id3-deficient mice, B6/B6 homozygosity in a locus on chromosome 12 close to the *Id2* gene is strongly correlated with the expansion of these γδ T cells. Although there is no protein coding sequence difference, the Id2 129 allele is expressed more in γδ T cells than the Id2 B6 allele, suggesting that in the 129 strain, higher Id2 expression is inhibiting the development of Vγ1.1^{+}Vδ6.3^{+} γδ T cells. In fact, bringing an Id2^{f} allele, which was created on the 129 genetic background, into the Id3 knockout mice can efficiently suppress the accumulation of Vγ1.1^{+}Vδ6.3^{+} γδ T cells. However, when both Id2 and Id3 are completely deleted, the Vγ1.1^{+}Vδ6.3^{+} γδ T cells do not expand more in the mouse than Id3 single knockout; on the contrary, the cells actually fail to accumulate, possibly due to attenuated proliferation and increased cell death induced by exceedingly high E protein activity (Zhang et al., 2013).

In order to independently clarify the possible inhibitory role of Id2 in γδ T cell development, I studied the Id2^{GFP} reporter mouse and the Id2$^{f/f}$CD4Cre^{+} single conditional knockout mouse, and I found that Id2 is indeed expressed in the mature γδ T cells in the thymus, as well as in the Vγ1.1^{+}Vδ6.3^{+} γδ T cells. Deletion of Id2 alone was sufficient to induce a moderate expansion of these populations, mainly through enhancing their survival. This results in an increase of γδ T cell numbers in the periphery. The results conclusively show that Id2 indeed is an inhibitor of γδ T cell

development, and support the idea that Id2 and Id3 act as dual safety mechanisms in regulating the population size of innate-like γδ T cells.

2.2 Materials and Methods

Mice

The $Id2^{GFP}$ (Rawlins et al., 2009) and $Id2^{f/f}$ (Niola et al., 2012) mice have been described previously and both backcrossed to B6 background for more than 10 generations. $Rosa^{ZsGeen/ZsGreen}$ mice on B6 background were purchased from Jackson Laboratories. CD4Cre transgenic mice on B6 background were purchased from Taconic. Animals were bred and maintained in the SPF facility managed by Duke University Division of Laboratory Animal Research. All animal procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Flow cytometry

The antibodies used in the flow cytometry analyses were as follows: anti-mouse CD4 (GK1.5), anti-mouse CD8a (53-6.7), anti-mouse B220 (RA2-6B2), anti-mouse/human CD44 (IM7), anti-mouse CD25 (3C7), anti-mouse NK-1.1(PK136), anti-mouse Ly-6G/Ly-6C(Gr-1) (RB6-8C5), anti-mouse CD11b(M1/70), anti-mouse TCRγ/δ(GL3), anti-mouse

TCR Vγ1.1 (2.11), anti-mouse CD24 (M1/69), anti-mouse TCRβ (H57-597), anti-mouse IFNγ (XMG1.2) and anti-mouse IL-17A (TC11-18H10.1) were purchased from Biolegend. The PE anti-mouse Vδ 6.3/2 (8F4H7B7) antibody, annexin V and the APC BrdU Flow Kit were purchased from BD Biosciences. 7-Aminoactinomycin D (7-AAD) was purchased from Life Technologies.

Single-cell suspensions were prepared from thymus, spleen and peripheral lymph nodes, and suspended in cold FACS buffer (1×PBS supplemented with 5% bovine calf serum). 1×10^6 cells were stained with antibodies in the dark at 4°C for 30 min. After washing with cold FACS buffer, cell suspensions were analyzed on a FACSCanto II flow cytometer (BD Biosciences). For intracellular staining, cells were stimulated with PMA (10 ng/mL) and ionomycin (1 μg/mL) in the presence of monensin (3μM) (all from Sigma-Aldrich) for 5 hours at 37℃, stained for surface markers and 7AAD, then fixed and permeabilized with the Cytofix/Cytoperm kit (BD), immediately followed by intracellular staining and flow cytometry analysis. FlowJo software (Tree Star) was used for data analysis. Cell sorting was performed with a FACS DiVa sorter (BD Biosciences).

In vitro stimulation of γδ T cells

Thymic GFP-negative TCRγδ^{+} cells from Id2$^{GFP/+}$ mice were sorted and cultured in OP9-DL1 cell covered wells with MEM-α medium, supplemented with 10% fetal bovine serum and 5 ng/mL IL-7, with or without 1 μg/mL anti-TCRγδ (clone UC7-13D5, Biolegend). Cells were harvested after 5 days for FACS analysis.

In vivo BrdU incorporation assays

1 mg of BrdU was i.p. injected to each mouse 15 hours before sacrificing the mouse. Cells were harvested and processed with the BrdU Flow Kit (BD Biosciences) according to manufacturer protocol.

Real-time PCR

γδ T cells (TCRγδ^{+}) were sorted from the mouse thymus, and their RNA was extracted with RNaqueous micro kit (Life Technologies). The RNA was reverse transcribed into cDNA with random hexamers and M-MLV reverse transcriptase (Life Technologies). Real-time PCR was performed with a Mastercycler ep realplex (Eppendorf). 18s rRNA was used as an internal control. The primer sequences are: PLZF-F, CCACCTTCGCTCACATACAG; PLZF-R, CACAGCCATTACACTCATAGGG; PLZF-probe, /56-FAM/TGCCGCAGA/ZEN/ACTCACACTCATATGG/3IABkFQ/; Itk-F,

GTGTTTGACTCCATCCCTCTC; Itk-R, CCCACTTCCCATATCTTAGCC; Itk-probe, /56-FAM/CGACTCCGC/ZEN/TATCCAGTTTGCTCC/3IABkFQ; 18s rRNAF, GTT CCT TTG GTC GCT CGC TCC TC; 18S rRNAR, GGC ACG GCG ACT ACC ATC GA.

Statistical analysis

Sample data was compared using Student's t test, and p value less than 0.05 was considered significant.

2.3 Results

2.3.1 Id2 is expressed in the mature γδ T cells

To investigate the role of Id2 in the development of γδ T cells, I first examined its expression in γδ T cells using the $Id2^{GFP}$ reporter mouse. This mouse contains a GFP gene knock-in to the endogenous Id2 locus. The expression of GFP reflects the Id2 promoter activity in individual cells. The knock-in allele does not express normal Id2 mRNA, so $Id2^{GFP/+}$ heterozygote mice were used to preserve Id2 function. The mouse was generated with 129 embryonic stem cells and later backcrossed to B6 background for more than 10 generations, but the expression level of Id2 should be similar to that observed in 129/sv mice.

In the thymus, γδ T cells develop from DN3 cells that successfully rearrange the γδ T cell receptor. The nascent γδ T cells are $TCR\gamma\delta^{+}CD24^{+}CD44^{low}$ ("immature"); some

of these cells will go through a maturation process in the thymus and become TCRγδ^{+}CD24^{-}CD44^{hi} ("mature") (Prinz et al., 2006). Most γδ T cells in the periphery are CD24^{-}, although they may have a variety of CD44 expression levels.

Analyzing these populations from Id2$^{GFP/+}$ mice, I found Id2 is not expressed in the precursors of γδ T cells (DN2, DN3) (**Fig 3A**). The expression is also not detectable in immature γδ T cells, but it is markedly increased in the mature γδ T cells. The γδ T cells that express T cell receptors using the Vγ1.1 and Vδ6.3 segments also expressed higher levels of Id2 compared to the γδ T cells that do not use both V segments (**Fig 3B**).

It has been shown that γδ T cells maturing in the thymus may have encountered their cognate antigen and received signal through their TCRs (Haks et al., 2005). The Vγ1.1^{+}Vδ6.3^{+} T cells have also been shown to be autoreactive (O'Brien et al., 1992). An increase of Id2 expression in the mature γδ T cells and in the Vγ1.1^{+}Vδ6.3^{+} T cells suggests that Id2 may be up-regulated by TCR signaling. To test this hypothesis, I sorted GFP negative, TCRγδ^{+} cells from the thymi of Id2$^{GFP/+}$ mice and cultured them with or without anti-TCRγδ antibody stimulation for 5 days. The TCR stimulation indeed resulted in higher Id2 expression (**Fig 3C**), supporting our hypothesis.

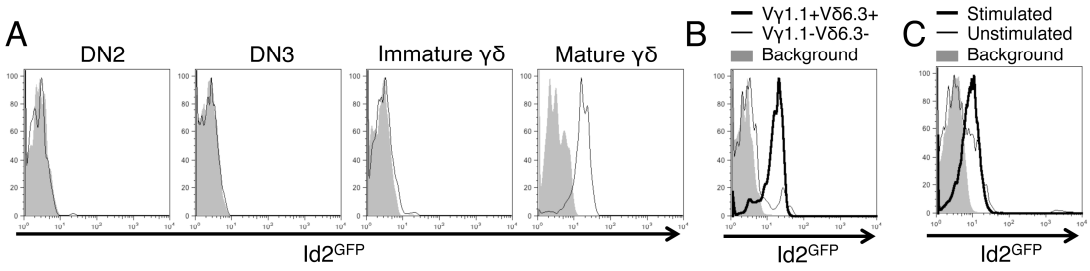

Figure 3: Id2 is expressed in thymic γδ T cells.

(A) Examination of Id2 expression in developing γδ T cells in the thymus with an $Id2^{GFP}$ reporter showed that Id2 is up-regulated at the mature stage. DN2: $Lin^{-}CD25^{+}CD44^{+}$. DN3: $Lin^{-}CD25^{+}CD44^{-}$. Immature: $TCR\gamma\delta^{+}CD24^{+}CD44^{low}$. Mature: $TCR\gamma\delta^{+}CD24^{-}CD44^{high}$. **(B)** Id2 expression is higher in the $V\gamma1.1^{+}V\delta6.3^{+}$ cells than in other γδ T cells. **(C)** In vitro culturing of sorted $Id2^{GFP}$ negative γδ T cells from the thymus for 5 days with IL-7 and anti-TCR γδ stimulation resulted in more significant up-regulation of Id2 compared to culturing with IL-7 alone. Data representative of 3 mice in each group.

2.3.2 Conditional Id2 deficiency results in increased numbers of γδ T cells in the periphery

In order to understand the functional significance of the observed Id2 expression in developing γδ T cells, I utilized the $Id2^{f/f}$ CD4Cre conditional Id2 deficiency mouse model. CD4Cre is only active in a minor population of DN2 and DN3 cells, but it is highly active in the mature TCRγδ^{+} populations (**Fig 4**), making it a suitable deletor to study Id2 function in γδ T cells.

In the spleen, the $Id2^{f/f}$ $CD4Cre^{+}$ mice contained a larger population of γδ T cells than the $Id2^{f/f}$ $CD4Cre^{-}$ mice, both in terms of percentage and absolute numbers (**Fig 5A-B**). This result clearly points out that Id2 is an inhibitor of γδ T cell development. The increase can be caused by an increase of thymic output or an increase of peripheral expansion. Although there appeared to be an increase in the percentage of γδ T cells in the thymus of $Id2^{f/f}CD4Cre^{+}$ mice, when the numbers of γδ T cells in the thymus were analyzed, no difference was observed between $Id2^{f/f}CD4Cre^{+}$ and $Id2^{f/f}CD4Cre^{-}$ mice (**Fig 5C**). The increase in γδ T cell percentage may reflect a decrease in numbers of other cell types, such as αβ T cells. This result indicates that Id2 deficiency does not directly increase numbers of thymic γδ T cells. However, Id2 may affect thymic γδ T cell output through other mechanisms. Previously, I found that mature γδ T cells in the thymus express higher levels of Id2. Mature γδ T cells from the thymus have been shown to have better survival in the periphery than immature γδ T cells (Tough and Sprent, 1998).

If deficiency of Id2 causes a change in the maturation of thymic γδ T cells, this can potentially contribute to a population size change of the peripheral γδ T cells. Therefore, I next performed more detailed analysis of thymic γδ T cells in the conditional knockout mice.

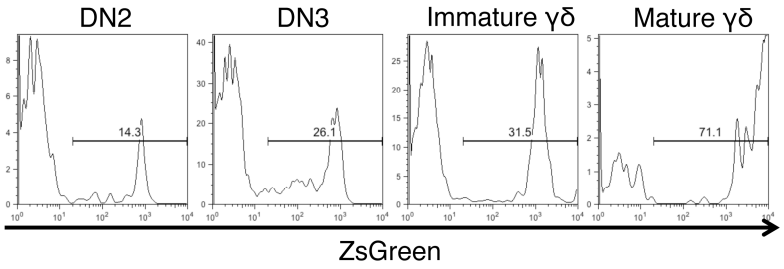

Figure 4: CD4Cre is active in the mature γδ T cells.

Thymocytes from CD4Cre^{+} Rosa$^{ZsGreen/+}$ mice were analyzed for ZsGreen expression. Cre activity will result in removal of a floxed transcription stop in the ZsGreen allele and induce permanent expression of ZsGreen, a green fluorescence marker. Although CD4Cre does not turn on ZsGreen in most DN2, DN3 and immature γδ T cells, it is highly active in mature γδ T cells. Data representative of 3 independent experiments.

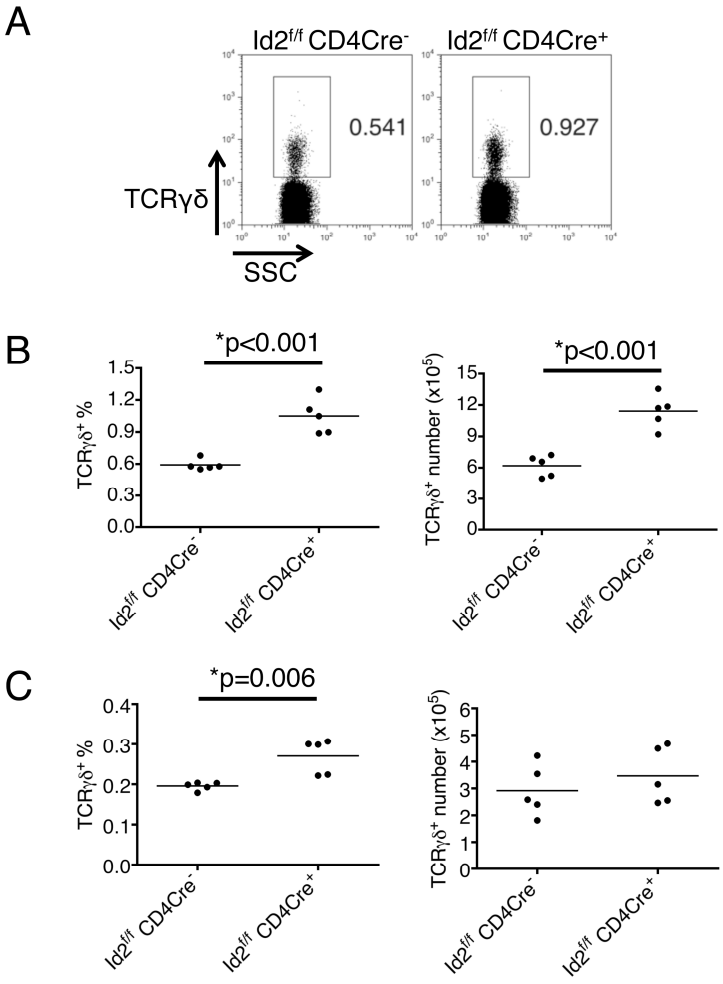

Figure 5: Conditional Id2 deficient mice have more γδ T cells in the peripheral lymphoid organ.

(A) Id2$^{f/f}$ CD4Cre^{+} mice have more γδ T cells in the spleen compared to Id2$^{f/f}$ CD4Cre^{-} mice. **(B)** Statistical plots show the percentage and number of γδ T cells in the spleen of mice in each group. **(C)** Same analysis showed a higher percentage of γδ T cells in the Id2$^{f/f}$CD4Cre^{+} mouse thymus but no increase in absolute numbers of the cells. Each dot represents one mouse.

2.3.3 Conditional Id2 deficiency increases the proportion of γδ T cells with a mature phenotype in the thymus

As expected, in the $Id2^{f/f}CD4Cre^{+}$ mice, I found a larger population of $CD24^{-}CD44^{hi}$ mature γδ T cells in their thymi (**Fig 6A**). This increased maturation can be one of the possible explanations of the larger population of γδ T cells in the periphery. I also found that $Id2^{f/f}CD4Cre^{+}$ mice have a larger population of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells in their thymi, another population normally expressing high levels of Id2 (**Fig 6B**). Unlike the Id3 knockout mice, the expansion of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells in the Id2 conditional knockout mice is moderate and does not contribute to more than half of the total γδ T cells, suggesting that Id2 deficiency may lead to expansion of multiple subsets of γδ T cells, and the expansion is not limited to the $V\gamma1.1^{+}V\delta6.3^{+}$ T cells only.

In addition to the surface marker analysis, functional analysis also showed that γδ T cells from $Id2^{f/f}CD4Cre^{+}$ mice are more mature. There was an increase of IFNγ-producing γδ T cells in their thymus, while the proportion of IL-17A-producing cells did not change (**Fig 6C**). Noticeably, the fluorescence intensity of intracellular staining of IL-17A appeared to be variable among different mice, but there was no significant difference between $Id2^{f/f}CD4Cre^{+}$ mice and $Id2^{f/f}CD4Cre^{-}$ mice (mean fluorescence intensity of IL-17A staining among the IL-17A positive cells: 84.3 ± 12.5 for Cre^{+} mice, 78.13 ± 42 for Cre^{-} mice, n=3 for each group)

It has been shown that γδ T cells which encounter their cognate antigens in the thymus will differentiate into IFNγ-producing cells, while those don't encounter antigens differentiate into IL-17A-producing cells (Jensen et al., 2008). It is possible that in those cells which encounter their cognate antigen and receive TCR signaling in the thymus, Id2 is up-regulated and inhibits their development; therefore, when Id2 is removed, the population expands.

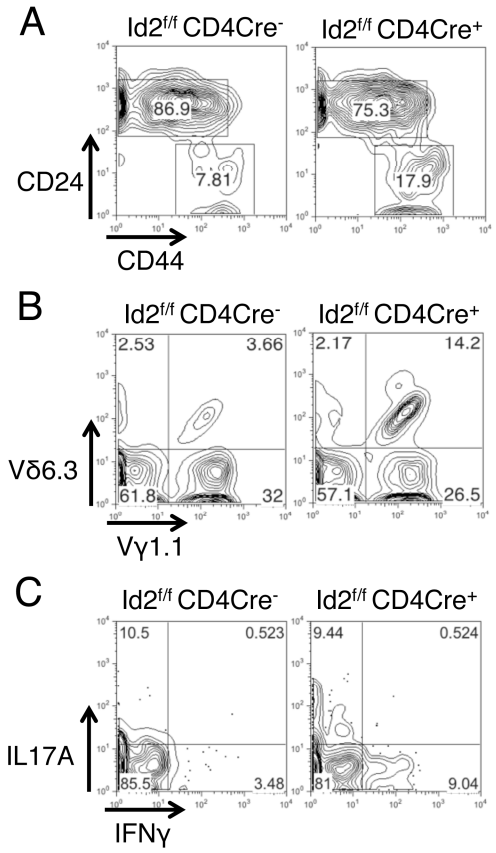

Figure 6: Conditional Id2 deficient mice have more mature γδ T cells and Vγ1.1^{+}Vδ6.3^{+} T cells in the thymus.

The proportion of CD24^{-}CD44^{hi} mature γδ T cells **(A)** and Vγ1.1^{+}Vδ6.3^{+} T cells **(B)** is higher in the Id2$^{f/f}$CD4Cre^{+} mouse thymus. **(C)** The percentage of IFNγ-producing population is also larger, but the IL-17A-producing population is not. Data representative of three independent experiments.

2.3.4 Conditional Id2 deficiency enhances the survival of γδ T cells in the thymus

To investigate why $Id2^{f/f}CD4Cre^{+}$ mice have more mature γδ T cells in their thymus, I considered two possibilities. The cells may have improved survival or increased proliferation in the absence of Id2 inhibition. I stained the mature γδ T cells with 7AAD and annexin V and found that cells from $Id2^{f/f}CD4Cre^{+}$ mice contain fewer $7AAD^{+}$ annexin V^{+} dead cells (**Fig 7A**). This indicates that the elevated level of Id2 expression normally observed in these cells promotes their apoptosis; in the absence of Id2, the cells gain a survival advantage. On the other hand, when I analyzed the proliferation of mature γδ T cells using the BrdU incorporation assay, I did not observe a difference between $Id2^{f/f}$ $CD4Cre^{+}$ and $Id2^{f/f}$ $CD4Cre^{-}$ mice (**Fig 7B**). This is in contrast to the previous report on the effect of Id3 deletion on $V\gamma1.1^{+}V\delta6.3^{+}$ T cells, in which case the cells gain a tremendous proliferation capacity (Zhang et al., 2013).

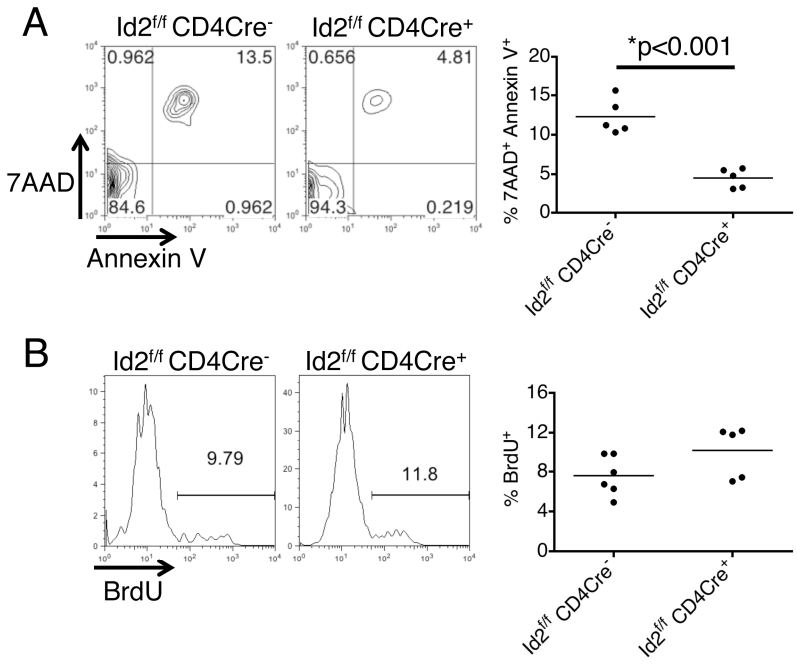

Figure 7: Id2 deficient mature γδ T cells have improved survival but no proliferation advantage.

(A) 7AAD/annexin V analysis showed that there is a smaller proportion of dead cells among mature γδ T cells in Id2$^{f/f}$CD4Cre^{+} mice. **(B)** BrdU incorporation assay did not show a difference of proliferating cell percentage between Id2$^{f/f}$CD4Cre^{+} mice and Id2$^{f/f}$CD4Cre^{-} mice. Each dot represents one mouse.

2.3.5 Id2 deficient mature γδ T cells express more *PLZF* and *Itk*

To characterize the Id2-deficient γδ T cells further, I surveyed their mRNA expression of several factors important for γδ T cell maturation and function. I found that Id2-deficient γδ T cells express more *PLZF*, a transcription factor important for innate-like γδ T cells and NKT cells, as well as more *Itk*, a kinase mediating TCR signaling (**Fig 8**). This result may be caused by an expansion of the $PLZF^{hi}$ and/or Itk^{hi} cell subset, or it can be caused by generally increased expression of the two genes on a per cell basis, or both. It has been shown that in NKT cells, PLZF is an important driver for the production of various cytokines such as IL-4, IFNγ and IL-17A (Alonzo and Sant'Angelo, 2011). Itk has been proposed to be a mediator of negative selection in γδ T cells; in the absence of Itk, potentially autoreactive γδ T cells can expand (Qi et al., 2011). The overall increase of PLZF and Itk expression among mature thymic γδ T cells in $Id2^{f/f}CD4Cre^{+}$ mice further suggests that this population may be not only larger in its size but also either contains more NKT-like and autoreactive cells, or be more NKT-like and autoreactive on a per cell basis, than those from wild type animals.

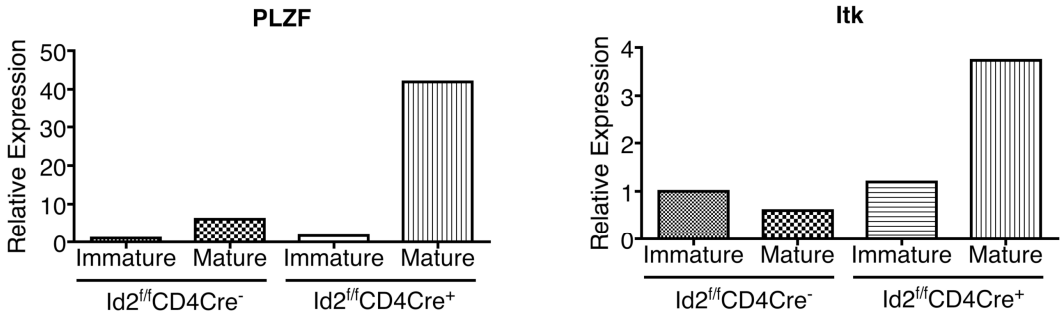

Figure 8: mRNA expression of *PLZF* and *Itk* in thymic γδ T cells.

Real-time PCR analysis of *PLZF* and *Itk* mRNA expression by thymic immature ($CD24^{+}CD44^{lo}$) and mature ($CD24^{-}CD44^{hi}$) $TCR\gamma\delta^{+}$ cells. Each bar indicates the average of triplicates from one sample pooled from two mice.

2.4 Discussion

The results of this study provide support for the genetic studies done by Baojun Zhang that Id2 indeed can function as an inhibitor of γδ T cell development. However, the phenotypic differences among wild type, Id3 knockout, Id2 single conditional knockout and Id2/Id3 double conditional knockout mice together create a complicated picture of E and Id function, defying a simple designation of these genes as pure inhibitors or enhancers of γδ T cell development.

I propose a dual safety mechanism model to describe the role of Id2 and Id3 specifically in the Vγ1.1$^+$Vδ6.3$^+$ γδ T cells (**Fig 9**). In this model, Id3 and Id2 are differentially regulated by the TCR signals. Egr is the major transcription factor acting between the TCR signal and Id3 in T cell development (Lauritsen et al., 2009). PLZF is a unique transcription factor involved in the development of innate-like lymphocytes such as iNKT and Vγ1.1$^+$Vδ6.3$^+$ γδ T cells (Dutta et al., 2013; Kreslavsky et al., 2009). Id2 has been shown to be activated by PLZF, which is a direct target of Egr2 in iNKT cell development (Gleimer et al., 2012). When both Id2 and Id3 are present, they respond to the TCR signal and keep E protein activity low, and consequently prevent the expansion of Vγ1.1$^+$Vδ6.3$^+$ γδ T cells. When Id3 is deleted, Id2 will assume a safety role to control E-protein activity. However, this safety role of Id2 is compromised by the hypomorphic allele of Id2 in the B6 background, allowing an increase in E protein activities to the

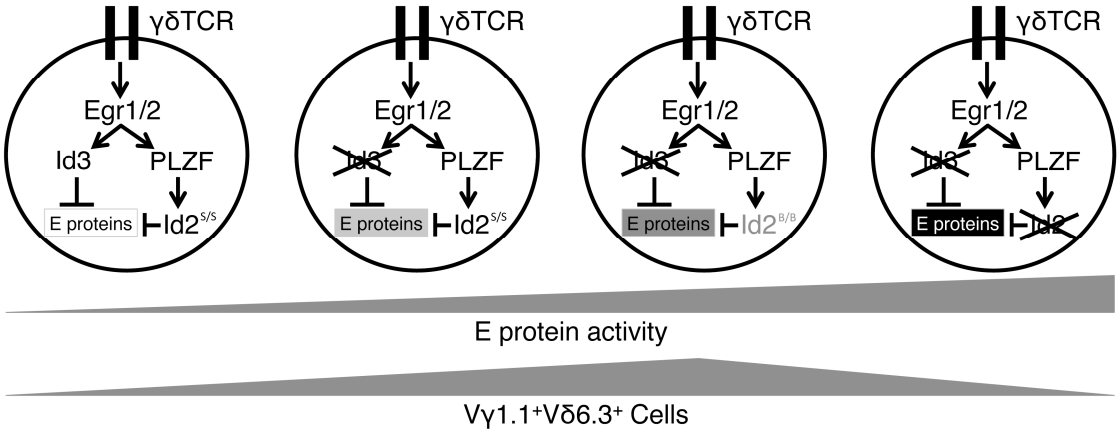

Figure 9: A schematic diagram of Vγ1.1^{+}Vδ6.3^{+} γδ T cell developmental control by Id2 and Id3.

In the developing thymus, γδ T cells that express the Vγ1.1 and Vδ6.3 TCR segments receive strong TCR signaling, up-regulating Id2 and Id3 through Egr1/2 and PLZF. The Id proteins inhibit activity of E proteins, affecting the survival and proliferation of Vγ1.1^{+}Vδ6.3^{+} γδ T cells. When Id3 is present, and Id2 is expressed from a more active allele, such as the one from the 129 genetic background (Id2^{S}, "strong"), E protein activity is very low and Vγ1.1^{+}Vδ6.3^{+} γδ T cell population size is small. If Id3 is absent, and Id2 is expressed from a less active allele, such as the one from the B6 background (Id2^{B}, "B6"), E protein activity becomes higher and the Vγ1.1^{+}Vδ6.3^{+} γδ T cells expand dramatically. However, if both Id2 and Id3 are completely absent, E protein activity becomes too high and again impairs the survival and proliferation of Vγ1.1^{+}Vδ6.3^{+} γδ T cells, limiting its population size.

optimal level for driving Vγ1.1^{+}Vδ6.3^{+} γδ T cell expansion. When both Id2 and Id3 are completely deleted, E protein activity becomes too high and again limits the Vγ1.1^{+}Vδ6.3^{+} γδ T cell population.

Our study indicated that the level of E protein activity, regulated by Id2 and Id3 expression levels, is crucial for γδ T cell development, especially during the "maturation" stage. Both very high and very low E protein activity can limit the accumulation of γδ T cells, especially the Vγ1.1^{+}Vδ6.3^{+} γδ T cells. Since Id proteins are up-regulated by TCR signaling, a developmental restraint imposed by high Id protein level and low E activity can be interpreted as a mechanism the body uses to limit the number of γδ T cells that can recognize self antigen in the thymus, reiterating the idea that Id2 and Id3 are "dual safety" involved in the negative selection of γδ T cells (Lauritsen et al., 2009). However, this "negative selection" seems to be affected by the age of the animal and TCR V segment usage. The Vγ1.1^{+}Vδ6.3^{+} γδ T cells, but not other autoreactive γδ T cells, dramatically expand during the neonatal period in Id3 deficient mice on B6 background. Why is this specific population particularly sensitive to Id protein regulation? One possibility is the presence of its cognate antigen. Vγ1.1^{+}Vδ6.3^{+} γδ T cells have been shown to recognize HSP60 of both mouse and Mycobacteria origin (O'Brien et al., 1992). Expression level of this antigen or other possible ligands of the Vγ1.1^{+}Vδ6.3^{+} γδ TCR may change in the thymus during development, thus making these

γδ T cells specifically prone to expand during the neonatal window, unless the Id proteins prevent them from doing so. Alternatively, the expression of Id proteins in response to TCR signaling may be different between cells generated in the neonatal period versus those generated in the adult stage, and different in cells utilizing other TCR V segments; mechanisms other than Id and E proteins may be more important in restraining autoreactive γδ T cells in those conditions, so they are less affected by Id protein deletion. Nevertheless, in the Id2 single conditional knockout mouse, γδ T cells other than those expressing Vγ1.1 and Vδ6.3 also expanded, indicating that Id2 is more broadly involved in the suppression of γδ T cell expansion.

However, our study also showed that total loss of Id2 and Id3 can impair γδ T cell proliferation and survival. Unrestricted E protein activity can lead to death of T cells, especially effector and memory T cells, which is well documented in the studies of peripheral CD4 and CD8 αβ T cells (Cannarile et al., 2006; Lin et al., 2012b). γδ T cells are considered innate-like cells, and many of them have an effector phenotype even in the thymus (Bonneville et al., 2010). Therefore, it is not surprising that they share the same requirement of Id protein activity with effector αβ T cells.

What is the physiological consequence of having a larger pool of autoreactive γδ T cells? The Id3 deficient mice spontaneously develop an autoimmune disease similar to human Sjogren's syndrome (Li et al., 2004). The large population of $V\gamma1.1^{+}V\delta6.3^{+}$ γδ T cells in these mice is potentially involved in the pathogenesis. However, a previous

report also showed that these cells can play a role in suppressing tissue inflammation (Carding and Egan, 2002). More tests are required to further clarify the impact of the expanded γδ T cell population in mice with Id protein deficiency. In the case of Id2, so far, I have not observed any signs of autoimmunity in the Id2 conditional knockout mice, despite their increased γδ T cell numbers. The impact of this enlarged population may become more prominent when the Id2 conditional knockout animal is challenged by triggers of autoimmune reactions that involve γδ T cells, such as imiquimod, which can induce a psoriasis-like skin disease (Cai et al., 2011). My hypothesis is the Id2 conditional knockout mice may demonstrate a stronger γδ T cell response to the stimulation.

3. Id2 is required for the CD4 T cell immune response in the development of experimental autoimmune encephalomyelitis

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(http://www.jimmunol.org/content/189/3/1400.long)

3.1 Introduction

The magnitude of a CD4 T cell response is a tightly controlled feature of the adaptive immune system. Upon activation through T cell receptor and co-stimulatory molecule signaling, a few antigen-specific CD4 T cells can proliferate to form a large pool of effector cells capable of performing immune functions. The expansion phase is then followed by contraction of the population to leave a small number of long-lived memory cells. Various intrinsic and extrinsic factors, including transcription factors and cytokines, have been implicated in regulating the T cell population size at each stage of this response (Taylor and Jenkins, 2011). For example, expression of the transcription factors T-bet (Joshi et al., 2007) and Blimp-1(Rutishauser et al., 2009) has been shown to

be associated with reduced T cell survival upon contraction, whereas IL-7 has been shown to promote T cell survival through this phase by promoting the expression of anti-apoptotic protein Bcl-2 (Kaech et al., 2003). However, why each individual T cell expresses different levels of these intrinsic factors, why each individual T cell responds differently to extrinsic factors and how these intrinsic-extrinsic factors cross-regulate each other are still not well understood.

As mentioned in Chapter 1, recently, the Inhibitor of DNA Binding (Id) proteins, including Id2 and Id3, have been identified to be important in the control of many aspects of T cell responses, including the T cell population size. In CD8 T cells, Id2 and Id3 have been shown to control the numbers of effector and memory cells, at least partially through promoting T cell survival (Cannarile et al., 2006; Ji et al., 2011; Yang et al., 2011). However, relatively little is known about the role of Id2 in CD4 T cell responses. One previous report has shown that Id2-deficient mice have increased Th2 dominance, but this difference was largely caused by the lack of a $CD8^+$ dendritic cell subset and therefore was not necessarily related to Id2 function in CD4 T cells (Kusunoki et al., 2003). Nevertheless, two other studies with double-positive thymocytes (Jones and Zhuang, 2007) and pro-T cell lines (Schwartz et al., 2006) showed that E protein transcription factors, the direct target proteins of Id2, may regulate genes important for CD4 T cell responses, including apoptosis-related genes *Bcl-2* and *Bim*, cell cycle-related genes *Rb* and *Cdk6*, and cytokine signal regulators *SOCS1* and *SOCS3*(Jones and Zhuang,

2007; Schwartz et al., 2006). *SOCS1* and *SOCS3* directly control the CD4 T cell response to multiple cytokines regulating effector and/or memory function and population size, such as IL-7, IL-6, IL-12 and IL-15 (Chen et al., 2000). Many of those cytokines can also regulate the expression of Id2 (Yang et al., 2011). Thus, Id2 may be involved in the cross-regulation of intrinsic and extrinsic factors for CD4 T cell population control. Thus far, studies based on Id2 knock-out mice cannot resolve these possibilities because the mice do not have normal development of lymph nodes (Yokota et al., 1999), and the model cannot separate CD4 T cell-intrinsic roles of Id2 from extrinsic ones.

To investigate how Id2 is involved in CD4 T cell responses, we studied Id2 conditional knock-out mice (Niola et al., 2012) with the experimental autoimmune encephalomyelitis (EAE) model, a CD4 T cell-dominant autoimmune disease model. EAE is a rodent model of human multiple sclerosis. By administering exogenous neuroautoantigens, a small number of pre-existing autoreactive CD4 T cells in the mice can be activated and induce central nervous system inflammation, demyelination and paralytic symptoms. The EAE model is an ideal tool to reveal potential roles of Id2 in many aspects of CD4 T cell responses, including T cell activation, differentiation, migration and population maintenance. With an Id2 reporter mouse model, we found that Id2 is dynamically regulated in the process of CD4 T cell activation. More strikingly, mice with T cell-specific Id2 deficiency are resistant to EAE, developing a smaller effector population in their peripheral lymphoid organs that fail to infiltrate the central

nervous system. This defect is at least in part due to reduced percentage of proliferating cells and increased death of effector CD4 T cells, and analysis of genes dysregulated in the absence of Id2 showed higher expression of *Bim* and *SOCS3* in these cells. These results establish the importance of Id2 in effector CD4 T cell population size control.

3.2 Materials and Methods

Animals

The $Id2^{hCD5/hCD5}$ reporter mouse model has been reported previously (Jones-Mason et al., 2012). Briefly, an IRES-driven, truncated human CD5 (hCD5) cDNA, without the sequence encoding the intracellular signaling domain, was knocked-in to the 3′ untranslated region of the Id2 gene. The $Id2^{f/f}$ mouse was used to generate conditional Id2 knock-out mice and has been described (Niola et al., 2012). The entire protein coding region of the Id2 gene was flanked by loxP sites. These mice were crossed with CD4-Cre transgenic mice (Taconic) to generate T cell-specific Id2-deficient mice (Δ/Δ, $Id2^{f/f}$ $CD4Cre^{+}$). The CD4Cre transgene-negative littermates were used as wild type controls (f/f, $Id2^{f/f}$ $CD4Cre^{-}$). All animal work was reviewed and approved by the Duke IACUC.

T cell culture and stimulation

Splenic $CD44^{low}CD62L^{+}$ naïve CD4 T cells were sorted and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 55 μM 2-mercaptoethanol. For TCR stimulation, cells were treated with plate-bound anti-CD3ε (1 μg/mL) and anti-CD28 (2.5 μg/mL) (both from Biolegend) plus IL-2 (2 ng/mL, PeproTech). For un-stimulated wells, cells were treated with IL-2 only.

Induction of EAE

EAE was induced in 6-week-old male mice by subcutaneous injection with 100 μg myelin oligodendrocyte glycoprotein35-55 (MOG35-55) peptide (MEVGWYRSPFSRVVHLYRNGK, United Peptide Corporation) emulsified in 100μL CFA (Sigma-Aldrich) containing 2 mg/ml of heat-killed Mycobacterium tuberculosis H37RA (Difco). Mice were also injected intraperitoneally with 200ng of pertussis toxin (List Biological Laboratories) on day 0 and day 2. Clinical signs of EAE were recorded daily using a 0 to 4 scoring system: 0, normal; 1, limp tail; 2, unsteady gait; 3, hind-limb paralysis; 4, four-limb paralysis.

Cell preparation and flow cytometry analysis

CD4 T cells were harvested from spleens and draining lymph nodes (inguinal and axillary). Mononuclear cells from the CNS were obtained as following: brain and spinal cord tissue were digested with 2.5 mg/mL collagenase D (Roche Applied Science) at 37℃ for 50 minutes. The tissue was then passed through a 70μm cell strainer and applied to Percoll gradient (30%/70%) centrifugation. Lymphocytes were collected from the interface.

Antibodies against CD4 (RM4-5), CD8α (53-6.7), CD44 (IM7), CD62L (MEL-14), IL-17A (Tc11-18H10.1), IFN-γ (XMG1.2) were from Biolegend. The antibody against human CD5 (hCD5, UCHT2) was from BD Bioscience. 7AAD was from Life Technologies. The mouse MOG38-49 I-A (b) tetramer was supplied by the NIH Tetramer Core Facility to identify the MOG-specific CD4 T cells. For surface staining, single-cell suspensions ($2x10^6$ cells) were stained for 15 minutes at 4℃. For tetramer staining, cells were stained for 3 hours at 37℃ as previously reported (Matsushita et al., 2008). For intracellular staining, cells were stimulated with PMA (10 ng/mL) and ionomycin (1 μg/mL) in the presence of monensin (3μM) (all from Sigma-Aldrich) for 5 hours at 37℃, stained for surface markers and 7AAD, then fixed and permeabilized with the Cytofix/Cytoperm kit (BD), immediately followed by intracellular staining and flow cytometry analysis. For BrdU staining, 1 mg of BrdU in D-PBS was injected intraperitoneally 15 hours before sacrificing the animal, and the staining was performed

with the BD BrdU Flow Kit according to manufacturer protocols. Cells were analyzed with a FACSCanto flow cytometer (BD) or sorted with a MoFlo cell sorter (Beckman Coulter).

Real-time PCR

Splenocytes were harvested from mice 9 days after EAE induction. CD4 T cells were enriched with the EasySep mouse $CD4^+$ T cell enrichment kit (Stemcell Technologies). MOG I-$A(b)^+CD4^+$ T cells were sorted, and total RNA from the cells were extracted followed by DNase I treatment using the RNAqueous micro kit (Life Technologies). Reverse transcription was performed with M-MLV reverse transcriptase (Life Technologies). The cDNA was used for real-time PCR with a Mastercycler ep realplex (Eppendorf). 18s rRNA was used as an internal control. The primer sequences are: Bcl2F, GGACTTGAAGTGCCATTGGTA; Bcl2R, GTTATCATACCCTGTTCTCCCG; Bcl2probe, /56-FAM/TGCGCCATC/ZEN/CTTCCCCGAAA/3IABkFQ/; BimF, GAGATACGGATTGCACAGGAG; BimR, CGGAAGATAAAGCGTAACAGTTG; Bimprobe, /56-FAM/TTCAGCCTC/ZEN/GCGGTAATCATTTGC/3IABkFQ/; SOCS1F, CTGCAGGAGCTGTGTCG; SOCS1R, CCCCACTTAATGCTGCGG; SOCS1probe, /56-FAM/CGCATCCCT/ZEN/CTTAACCCGGTACTC/3IABkFQ/; SOCS3F, CCTATGAGAAAGTGACCCAGC; SOCS3R, TTTGTGCTTGTGCCATGTG; SOCS3probe,

/56-FAM/CCCCTCTGA/ZEN/CCCTTTTGCTCCTT/3IABkFQ/; 18s rRNAF, GTT CCT TTG GTC GCT CGC TCC TC; 18S rRNAR, GGC ACG GCG ACT ACC ATC GA.

3.3 Results

3.3.1 Activated CD4 T cells express higher levels of Id2 than naive CD4 T cells

We used an $Id2^{hCD5/hCD5}$ reporter mouse model, in which surface hCD5 expression can be used as a marker for cellular Id2 expression (Jones-Mason et al., 2012), to first investigate the expression of Id2 in different subsets of CD4 T cells. In the spleen of naïve mice, we found that $CD44^{hi}CD62L^{-}$ effector memory-like CD4 T cells expressed higher levels of hCD5 than the $CD44^{hi}CD62L^{+}$ central memory-like or the $CD44^{low}CD62L^{+}$ naïve CD4 T cells (**Fig 10A**), suggesting that Id2 expression is correlated with previous T cell activation. Because the exact activation history of the pre-existing effector memory-like CD4 T cells is not defined in these mice, we next sorted $CD44^{low}CD62L^{+}$ naïve CD4 T cells and cultured them with anti-CD3/anti-CD28 stimulation plus IL-2 or with IL-2 only. After 48 hours of culture, the TCR-stimulated cells expressed higher levels of hCD5 than the un-stimulated cells (**Fig 10B**), showing that Id2 is dynamically regulated in the process of T cell activation. In order to confirm this finding in vivo, we induced EAE, a CD4 T cell-mediated autoimmune disease, in the $Id2^{hCD5/hCD5}$ reporter mice. Nine days after subcutaneous immunization with MOG peptide, we analyzed the MOG-specific CD4 T cells, as well as IL-17A and/or IFNγ-producing CD4 T cells from the draining lymph nodes. We again found that these cells expressed higher levels of hCD5 than the

non-MOG-specific or non-cytokine-producing CD4 T cells (**Fig 10C-D**). The in vitro and in vivo findings of Id2 up-regulation after CD4 T cell activation suggest that Id2 may play a role during the activation process and/or in the maintenance of the activated T cells.

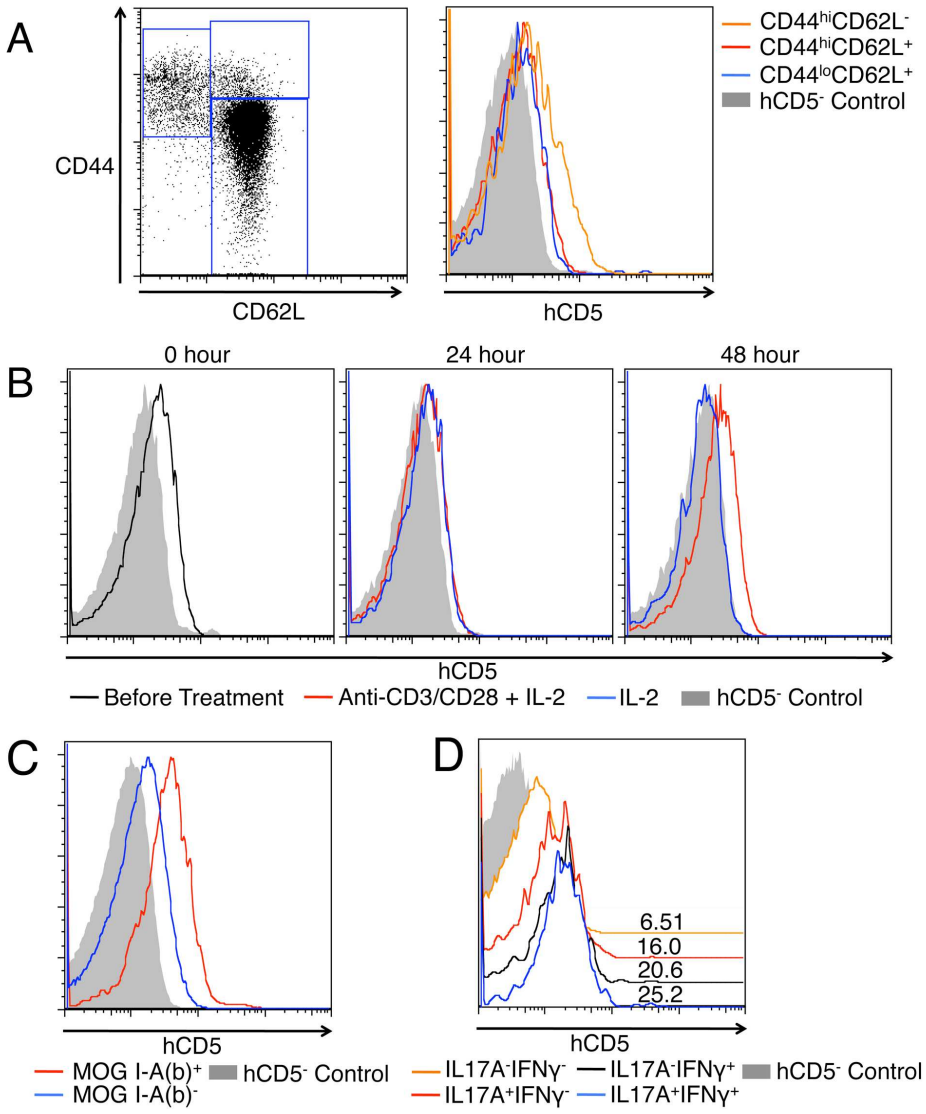


Figure 10: Activated CD4 T cells express higher levels of Id2 than naïve CD4 T cells

(A) The expression of hCD5 by subsets of splenic CD4 T cells from Id2$^{hCD5/hCD5}$ reporter mice was analyzed. Left: gating of the subsets. Right: histograms showing the hCD5 expression of CD44^{hi}CD62L^{-} (orange), CD44^{hi}CD62L^{+} (red) and CD44^{low}CD62L^{+} (blue) CD4 T cells. **(B)** Splenic CD44^{low}CD62L^{+} naïve CD4 T cells from Id2$^{hCD5/hCD5}$ reporter mice were sorted and cultured with anti-CD3/anti-CD28 antibodies plus IL-2 (red) or with IL-2 only (blue). Histograms show their hCD5 expression before, 24 hours and 48 hours after culture. **(C)** Expression of hCD5 by MOG I-A (b)$^{+}$ (red) or MOG I-A (b)$^{-}$ (blue) CD4 T cells isolated from draining lymph nodes of Id2$^{hCD5/hCD5}$ reporter mice 9 days after EAE

induction. **(D)** Expression of hCD5 by IL-17A$^+$IFNγ^+ (blue), IL-17A$^-$IFNγ^+ (black), IL-17A$^+$IFNγ^- (red) and IL-17A$^-$IFNγ^- (orange) CD4 T cells from the same lymph nodes as C. Numbers above histograms indicate mean fluorescence intensity. Shaded histograms in each plot showed hCD5 background of comparable populations of wild type CD4 T cells. All plots are representative of three independent experiments.

3.3.2 Mice with T cell-specific Id2 deficiency do not develop EAE

To test the functional significance of the up-regulation of Id2 expression in CD4 T cells during immune responses in vivo, we utilized the EAE model to compare the control (f/f, $Id2^{f/f}$CD4Cre-) mice versus T cell-specific Id2-deficient (Δ/Δ, $Id2^{f/f}$ $CD4Cre^{+}$) mice. Both mice have grossly normal inguinal (**Fig 11A**) and axillary (data not shown) lymph node development. In the naïve mice, despite a small reduction in total thymocyte numbers, the T cell-specific Id2-deficient mice have similar thymocyte development and splenic CD4 T cell composition compared to the control mice (**Fig 12A to H**). Similarly, no difference was observed with inguinal and axillary lymph node cell analysis (data not shown). The Id2-deficient CD4 T cells are also able to differentiate into IL-17-producing Th17 cells in vitro, with ability to survive and proliferate comparable to the control cells (**Fig 12I**). After immunization with MOG peptide, all control mice developed paralytic symptoms characteristic of EAE; however, none of the T cell-specific Id2-deficient mice developed the disease (**Fig 11B**). Because the development of disease symptoms in the EAE model is critically dependent on CD4 T cell infiltration into the CNS, we analyzed the CNS tissue from the mice 15 days after EAE induction, when the disease severity was at its peak in control mice. While we found a significant number of CD4 T cells in the brain and spinal cord of control mice, nearly no CD4 T cell infiltration was observed in the CNS of T cell-specific Id2-deficient mice (**Fig 13A-B**). The CD4 T cells found in the CNS of control mice consist of a significant proportion of MOG-

specific cells, as well as IL-17 and/or IFNγ producing cells, while the few CD4 T cells found in the CNS of T cell-specific Id2-deficient mice do not contain these populations (**Fig 13C-D**). Together, the complete resistance to the disease and the absence of T cell infiltration in the CNS showed that Id2 expression is indeed functionally relevant to CD4 T cell responses in vivo, and Id2 deficiency may cause a significant defect in CD4 T cell response to MOG immunization, possibly early in the initiation phase of the disease.

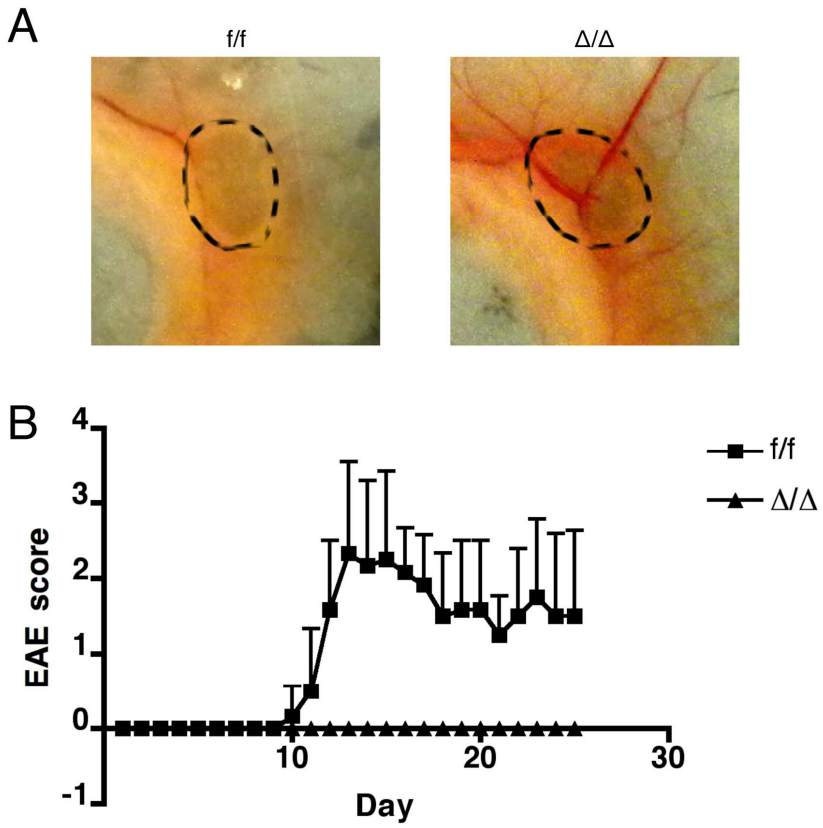


Figure 11: Mice with T cell-specific Id2 deficiency are resistant to EAE

(A) Representative photographs of inguinal lymph nodes (circled by dashed lines) from control (f/f, $Id2^{f/f}CD4Cre^{-}$) and T cell-specific Id2-deficient (Δ/Δ, $Id2^{f/f}CD4Cre^{+}$) mice. n=5 for each group. **(B)** EAE was induced in six-week-old mice, and their disease scores were recorded daily. Score 0: normal, 1: limp tail, 2: unsteady gait, 3: hind limb paralysis, 4: four limb paralysis. f/f, n=6; Δ/Δ, n=7. Error bars indicate S.D.

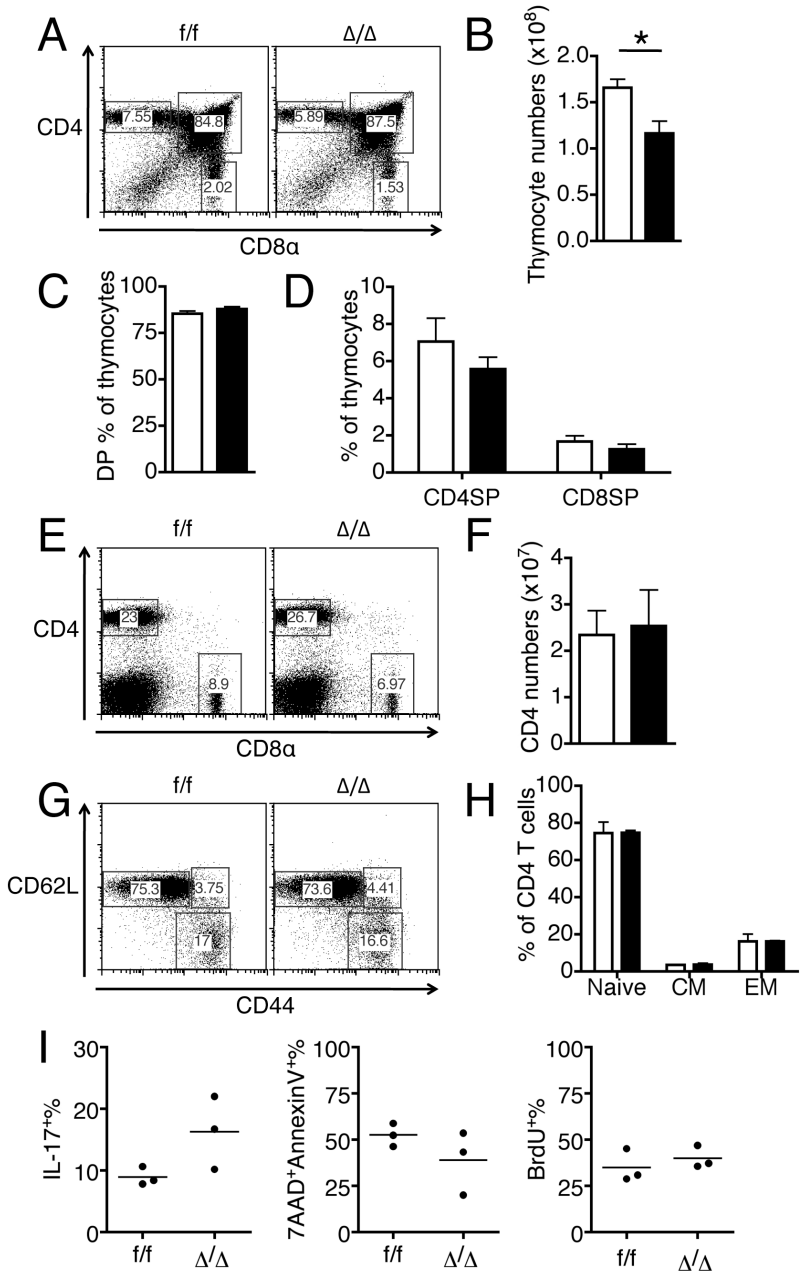


Figure 12: Mice with T cell-specific Id2 deficiency have similar thymocyte development, splenic CD4 T cell composition and in vitro Th17 differentiation compared to control mice

(A) Representative thymocyte flow cytometry analysis shows percentage of double positive and single positive thymocytes in control (f/f) and T cell-specific Id2-deficient mice (Δ/Δ). Bar graphs compare **(B)** total thymocyte numbers, **(C)** percentage of double

positive cells and **(D)** percentage of CD4 single positive (CD4SP) and CD8 single positive (CD8SP) cells between the two groups. **(E)** Representative splenocyte flow cytometry analysis shows percentage of $CD4^+$ and $CD8^+$ T cells. **(F)** Numbers of total splenic $CD4^+$ cells. **(G and H)** Representative flow cytometry analysis and bar graph show the percentage of subsets of splenic $CD4^+$ T cells. **(I)** Analysis of $CD4^+$ T cells 8 days after *in vitro* culture in Th17 skewing condition. Splenic $CD4^+$ cells from naïve control and T cell-specific Id2-deficient mice were magnetically enriched and cultured in the presence of anti-CD3 (5 μg/mL), anti-CD28 (1 μg/mL), TGF-β (1 ng/mL), IL-6 (50 ng/mL), IL-23 (50 ng/mL), IL-1β (20 ng/mL), anti-IFNγ (10 μg/mL) and anti-IL-4 (10μg/mL). n=3 for each group in each graph. *$p < 0.05$. Error bars indicate S.D.

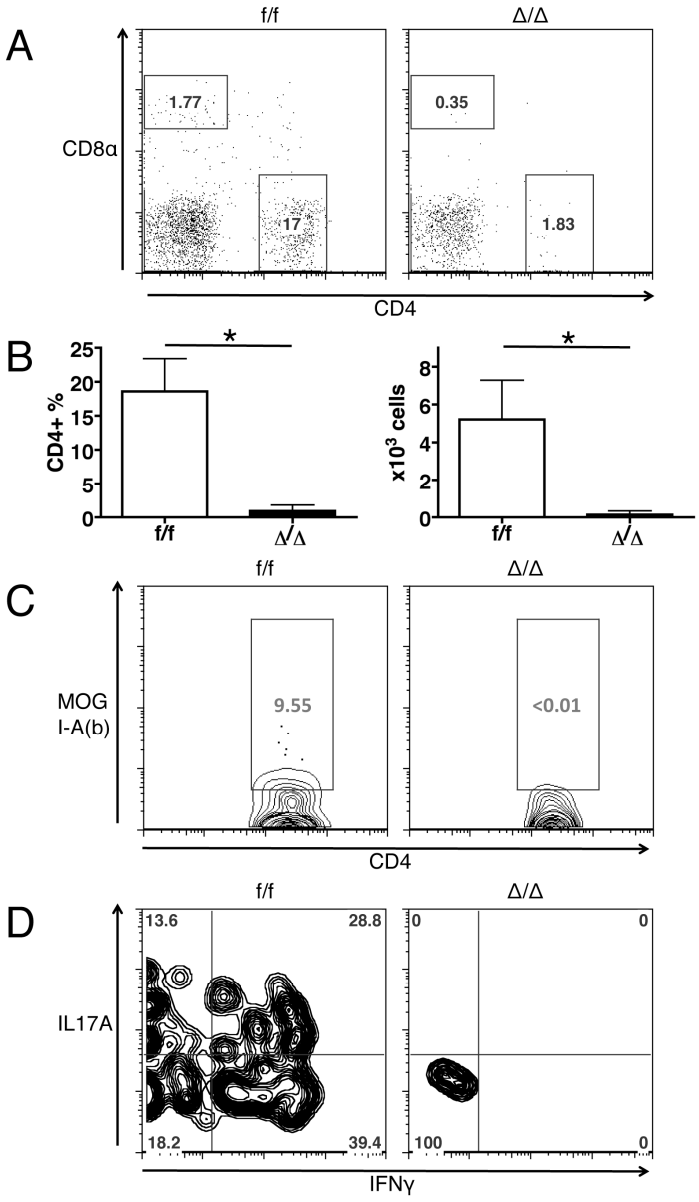


Figure 13: Mice with T cell-specific Id2 deficiency do not recruit CD4 T cells into the CNS 15 days after EAE induction

(A) Representative plots of CD4 and CD8α staining of CNS infiltrating cells. **(B)** Percentage and number of CD4 T cells in the CNS. n=3 for each group. *$p<0.05$. Error bars indicate S.D. **(C)** Representative plots of MOG I-A(b) staining of CNS infiltrating $CD4^+$ cells. **(D)** Representative plots of IL-17A and IFNγ staining of CNS infiltrating $CD4^+$ cells.

3.3.3 EAE induction generates a smaller pool of effector CD4 T cells in mice with T cell-specific Id2 deficiency

Two possibilities may lead to the absence of CD4 T cell infiltration to the CNS in mice with Id2 deficiency: the effector CD4 T cells may develop in the periphery normally, but fail to migrate to the CNS, or the development of the effector T cells may be defective. To distinguish these possibilities, we next examined the CD4 T cell response in peripheral lymphoid organs of the mice 9 days after immunization, when control mice started to show paralytic symptoms. Compared to control mice, the percentage and number of MOG-specific CD4 T cells was lower in the T cell-specific Id2-deficient mice both in the spleen and draining lymph nodes (**Fig 14A**). Intracellular cytokine staining also revealed a decrease of splenic cytokine-producing CD4 T cell populations in the T cell-specific Id2-deficient mice (**Fig 14B**), especially the IL-17A$^+$IFN-γ^+ cells, which have been reported to be especially encephalitogenic (Kebir et al., 2009). These results correlate with the higher expression of Id2 seen in these populations in the Id2$^{hCD5/hCD5}$ model (**Fig 10C-D**). It is noteworthy that residual MOG-specific and cytokine-producing CD4 T cells can be detected in the T cell-specific Id2-deficient mice, suggesting that these T cells have not totally lost their capability of activation and differentiation; presence of these cells in the spleen but not in the CNS also suggests that they have entered circulation but possibly failed to enter CNS. Several adhesion molecules and chemokine receptors have been shown to be important for the migration

of T cells in EAE, including α4β1 integrin (Rice et al., 2005) and CCR6 (Reboldi et al., 2009). We examined the expression levels of the two molecules by MOG-specific T cells from mice 9 days after EAE induction but found no difference between Id2-deficient and control cells (**Fig 15**). Therefore, the absence of Id2-deficient CD4 T cells in CNS is unlikely caused by change in the expression of α4β1 integrin or CCR6.

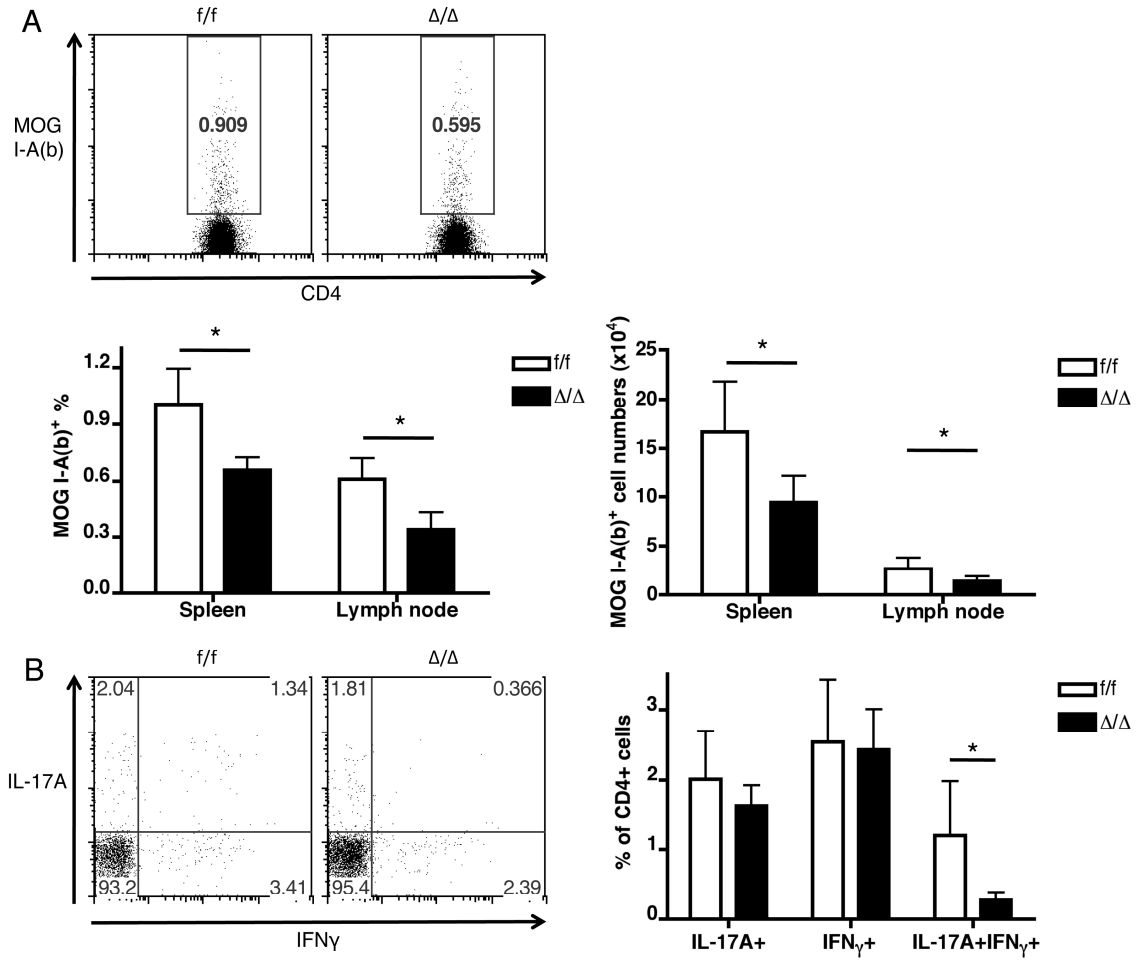


Figure 14: Mice with T cell-specific Id2 deficiency have a smaller pool of effector CD4 T cells in the secondary lymphoid organs 9 days after EAE induction

(A) Representative plots and bar graphs showing percentages and numbers of MOG-specific cells among CD4 T cells from spleens of control (f/f) and Id2 conditional knock-out (Δ/Δ) mice. Similar changes were observed in the lymph nodes as shown in the bar graphs. **(B)** Representative plots and bar graphs of cytokine-producing CD4 T cells from spleens of control (f/f) and Id2 conditional knock-out (Δ/Δ) mice. n≥3 for each group. *p < 0.05. Error bars indicate S.D.

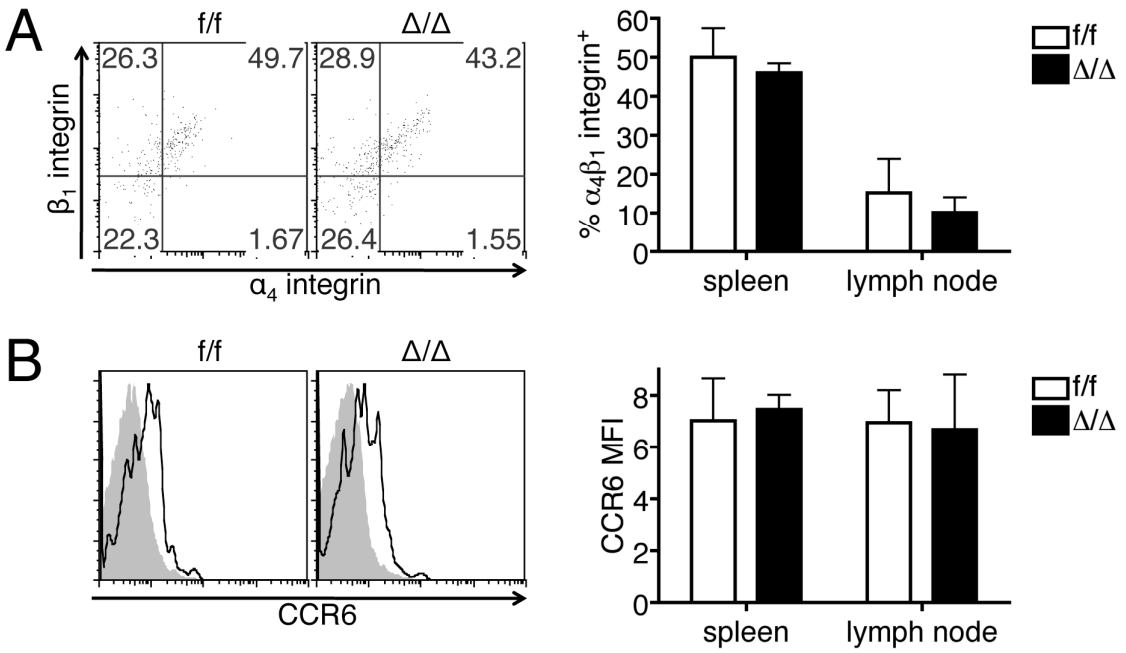


Figure 15: Id2-deficient MOG-specific CD4 T cells have similar expressions of α4β1 integrin and CCR6 compared to control cells

(A) Representative splenocyte plots and bar graphs showing the expression of α4β1 integrin by MOG-specific cells from spleens and lymph nodes from mice 9 days after EAE induction. **(B)** Representative splenocyte plots and bar graphs showing the expression of CCR6 by the same MOG-specific cell populations. Shaded histogram showed CCR6 staining of total CD4 T cells. n=3 for each group. Error bars indicate S.D.

3.3.4 Id2-deficient CD4 T cells show reduced percentage of proliferating cells and increased cell death

A smaller population of effector T cells may be the result of reduced cell proliferation or increased cell death. We examined the proliferation of MOG-specific CD4 T cells in the expansion phase of the immune response (6 days after EAE induction) by in vivo BrdU labeling. We found a significant decrease of percentage of $BrdU^+$ cells from the draining lymph nodes of T cell-specific Id2-deficient mice compared to the control mice (**Fig 16A**).We next compared cell death between control and Id2-deficient CD4 T cells with 7AAD staining. Corresponding to their reduced population size, we found increased cell death in MOG-specific CD4 T cells as well as IL-$17A^+$IFN-γ^+ CD4 T cells from T cell-specific Id2-deficient mice 9 days after EAE induction (**Fig 16B-C**). Repeating the experiments with a fixable Live/Dead stain (Life Technologies) generated similar results (data not shown). These findings indicate that Id2-deficient CD4 T cells, after activation, suffer from reduced proliferation, increased cell death, form a smaller effector cell population, and are unable to induce EAE.

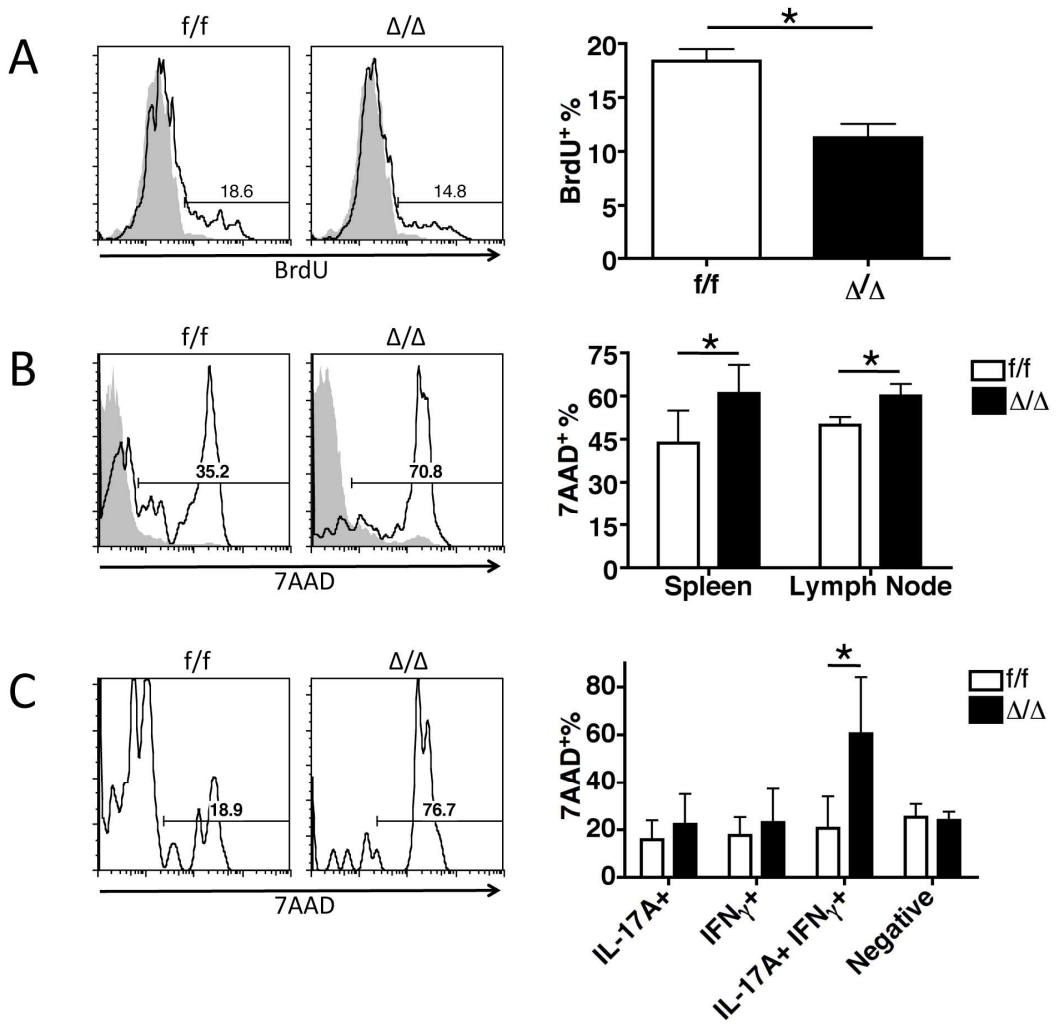


Figure 16: Id2-deficient CD4 T cells show reduced percentage of proliferating cells and undergo increased cell death

(A) Proliferating cells were labeled in vivo with BrdU 6 days after EAE induction. Cells from draining lymph nodes were analyzed 15 hours after BrdU injection. Line histogram: MOG I-A (b)$^+$ CD4+ cells; shaded histogram: MOG I-A (b)$^+$ CD4$^+$ cells from mice not injected with BrdU. **(B and C)** Cell death of the Id2-deficient MOG-specific CD4 T cell and IL-17A$^+$IFNγ^+ CD4 T cell populations 9 days after EAE induction were shown by 7AAD staining. **(B)** Line histogram: splenic MOG I-A (b)$^+$ cells; shaded histogram: MOG I-A (b)$^-$ cells. **(C)** Line histogram: splenic IL-17A$^+$IFNγ^+ CD4 T cells. Bar graph shown is analysis of splenic CD4 T cells. n≥3 for each group. *$p < 0.05$. Error bars indicate S.D.

3.3.5 Id2-deficient CD4 T cells express higher levels of *Bim* and *SOCS3*

Two possible mechanisms may explain how Id2 affects the CD4 T cell population size: direct control of apoptosis or inhibition of cytokine signaling. Both anti-apoptotic and pro-apoptotic genes, such as *Bcl-2* and *Bim*, have been reported to be altered in Id2-deficient CD8 T cells (Cannarile et al., 2006). We used real-time PCR to examine their expressions in splenic MOG-specific CD4 T cells from animals 9 days after EAE induction, and we found that the expression of the pro-apoptotic *Bim* was increased in Id2-deficient CD4 T cells but not *Bcl-2* (**Fig 17A**). This result correlates with the increased cell death of this population and is compatible with a previous report that E proteins can up-regulate *Bim* expression (Schwartz et al., 2006).

In addition to direct regulation of apoptosis, Id2 may also regulate cell survival and other aspects of T cell responses through regulation of cytokine signaling. Two important suppressors of cytokine signaling in T cells, *SOCS1* and *SOCS3*, have been reported to be up-regulated by E proteins (Jones and Zhuang, 2007; Schwartz et al., 2006). In particular, increased SOCS3 can inhibit cytokine signaling through STAT3, an important signal transduction mediator of pro-inflammatory cytokines such as IL-6 (Baker et al., 2009). T cell-specific STAT3 knock-out mice are also resistant to EAE and also demonstrate a reduced size of IL-17-producing effector CD4 T cell population (Liu et al., 2008). We examined the expression of *Socs1* and *Socs3* in Id2-deficient MOG-specific CD4 T cells by real-time PCR and found that *Socs3* expression was profoundly

up-regulated compared to control CD4 T cells; there was also a trend toward increased *Socs1* expression in these cells (**Fig 17B**). These results indicate that Id2 is required for the maintenance of the effector CD4 T cell population size through regulating the expression of genes related to apoptosis control and cytokine signaling.

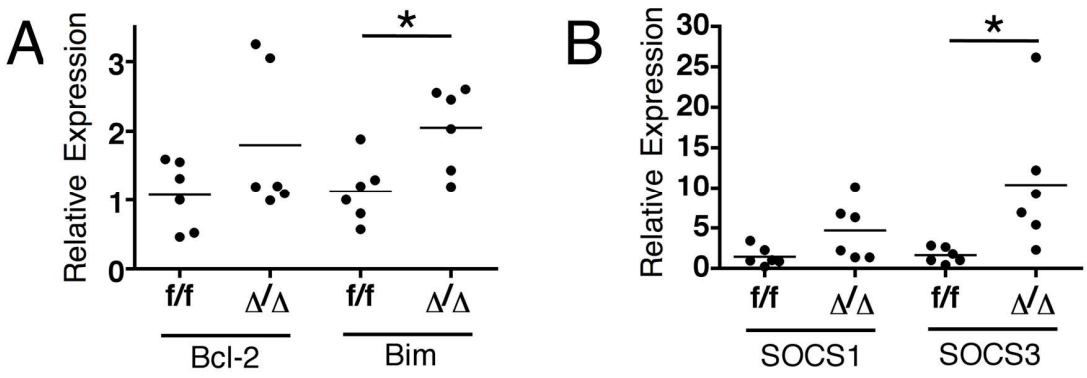


Figure 17: Id2-deficient MOG-specific CD4 T cells express higher levels of *Bim* and *Socs3* mRNA

Real-time PCR analysis of *Bcl-2, Bim* (A), *Socs1 and Socs3* (B) mRNA expression by sorted Id2-deficient MOG I-A (b)$^+$ CD4 T cells from spleens of mice 9 days after EAE induction, normalized to their control counter parts. Each dot represents an individual animal. *$p < 0.05$

3.4 Discussion

Once CD4 T cells get activated in an immune response, both intrinsic and extrinsic factors contribute to the maintenance and regulation of the effector T cells, controlling the size of the population and the function of individual cells. Here we demonstrate that transcriptional regulator Id2, previously best known for its role in the development of various lineages of hematopoietic cells (Kee, 2009), is also an important regulator of CD4 T cell responses, especially in the Th17-mediated EAE disease model.

In the absence of Id2, the population size of MOG-specific CD4 T cells is reduced. The cytokine-producing CD4 T cells are also reduced; specifically, we found that IL-17A$^+$IFNγ^+ CD4 T cells almost completely disappeared in the T cell-specific Id2-deficient mice. The findings implied that this population is particularly dependent on Id2 for its formation and/or survival. Previous studies reported that this population is more encephalitogenic and has a stronger propensity to migrate into the central nervous system (Kebir et al., 2009). In a tumor model, the IFNγ production capacity of Th17 cells has also been shown to be crucial for their anti-tumor activity (Muranski et al., 2011). These reports suggest that the IL-17A$^+$IFNγ^+ CD4 T cells may be important effector cells of a Th17-mediated immune response. They also help explain that, despite relatively comparable development of IL-17A$^+$ or IFNγ^+ CD4 T cells in the peripheral lymphoid organs of the T cell-specific Id2-deficient mice, and a reduced but still significant

population of MOG-specific CD4 T cell, no CNS infiltration or disease symptoms developed in these mice. Why this population is particularly dependent on Id2, and whether the population can be specifically targeted for controlling Th17-mediated autoimmune diseases, remain important questions to be addressed.

One possible explanation lies in Id2-mediated regulation of SOCS3. By removing the inhibitor Id2, SOCS3 expression increases. SOCS3 can suppress signaling of many Th17-related cytokines, including IL-21 and IL-23, through its association with the cytokine receptor and inhibition of JAK kinases, reducing the phosphorylation and activation of the downstream signal transduction molecule STAT3 (Baker et al., 2009). IL-21 production is up-regulated in Th17 cells upon IL-6 stimulation, and it can up-regulate the expression of IL-23 receptor by Th17 cells, increasing the cellular responsiveness to IL-23 and thus "stabilize" Th17 cells (Spolski and Leonard, 2008). IL-23 signaling is critical for the development of IL-17$^+$IFNγ^+ as well as IL-17$^-$IFNγ^+ cells from IL-17$^+$ cells during chronic inflammation, such as in EAE (Hirota et al., 2011). In the absence of Id2, these cytokine signaling processes may be impaired by SOCS3, thus impairing Th17 maintenance and evolution. In fact, T cell-specific STAT3 deficient mice are also resistant to EAE and experimental autoimmune uveitis (EAU) (Liu et al., 2008). These mice also have significantly reduced IL-17A$^+$IFNγ^+ CD4 T cells in the periphery. The phenotype similarity between STAT3-deficient CD4 T cells and Id2-deficeint CD4 T cells, and the up-regulation of SOCS3 in the latter, strongly suggests that Id2 influence

CD4 T cells through their regulation of cellular responses to cytokines. However, because E proteins and Id proteins regulate many genes, it is possible that other important pathways are also affected in the Id2-deficient CD4 T cells. A gene expression profiling experiment should provide us with a comprehensive overview of these potentially Id2-regulated pathways.

The current study focuses on the role of Id2 in the effector stage of the CD4 T cell immune response. Whether Id2 plays a role in the formation of CD4 T cell memory is also an important question. Yang et al previously showed that Id2 is important for the formation of short-lived effector memory CD8 T cells in a Listeria infection model (Yang et al., 2011). The possibility that Id2 contributes to the survival of effector memory CD4 T cells can be tested in the future with a variety of prime-boost immunization models or infection-rechallenge models. In addition, as shown in Chapter 2, Id2 also plays a significant role in the development of γδ T cells, and CD4Cre is capable of deleting Id2 in γδ T cells. It is likely that the disturbance of Id2 function in γδ T cells also influenced the disease resistance demonstrated by the Id2 conditional knockout mice in this chapter. Whether γδ T cells get activated in the periphery of these mice, how well is their population maintained, and whether Id2-deficient γδ T cells can invade the CNS, require further studies to clarify.

We have shown in this study that mice with T cell-specific Id2 deficiency do not develop EAE. However, whether removing Id2 after the disease already develops, such

as using chemical inhibitors of Id2 or inducible Id2 deletion models, can alter the disease course, is another interesting question. We predict that removal of Id2 after T cell activation should also be effective in inhibiting the T cell response, possibly by shrinking the effector T cell population through increased cell death. If confirmed, modulation of E protein-Id protein activity may become a possible direction of the development of future immune suppressive treatments.

4. Id3 inhibits tumor genesis driven by the EμMyc transgene in the lymphoid system

4.1 Introduction

Burkitt's lymphoma is a kind of homogeneous, medium sized B cell lymphoma that is the most rapidly progressive human tumor across all organ systems (Longo, 2011). The tumor size can double every 24 to 48 hours because almost 100% of the tumor cells are proliferative. The disease can also present in the form of leukemia. It has such an aggressive behavior probably because it originates from germinal center B cells, which naturally proliferate quickly and undergo somatic hypermutations for the selection and expansion of high affinity antibody-producing cells.

There are three different kinds of Burkitt's lymphoma: endemic, sporadic and immunodeficiency-associated. The endemic form is mostly found in Africa and is associated with Ebstein-Barr Virus infection. The immunodeficiency-associated form is commonly seen in patients infected with HIV. The sporadic form has no identifiable cause, and it typically occurs in children or young adults, presenting as peripheral lymphadenopathy or an intraabdominal mass. The sporadic form is a rare disease in Western countries, with an incidence of 3 per 1,000,000 people per year (Longo, 2011). Regardless of the kind, all Burkitt's lymphoma can be rapidly lethal if not treated quickly. Although modern combination chemotherapy can cure up to 70-80% of patients with Burkitt's lymphoma, the chemotherapy agents used are highly toxic, and a small proportion of patients still have resistant diseases or relapses (Molyneux et al., 2012).

Therefore, further investigation of the disease is needed to design new treatments and to improve care of these patients.

The sporadic form of Burkitt's lymphoma has long been associated with the t(8;14) chromosome translocation event, which brings the proto-oncogene c-Myc together with immunoglobulin heavy chain (IgH) gene and leads to over-expression of c-Myc in B cells. This is thought to contribute to the hyper-proliferative capacity of the tumor cells. Myc is a basic helix-loop-helix zipper (bHLH-Zip) protein and binds to DNA with its partner MAX (another bHLH-Zip protein), and together they recognize target sequences containing E box consensus sequences (CACGTG), similar to those recognized by E proteins (Blackwood and Eisenman, 1991). Because E box consensus sequences are present in a large number of genes, Myc has been proposed to be a global regulator of chromatin and gene expression, and cells that express more Myc have higher transcription activity in general and contain more RNA per cell (Loven et al., 2012). This results in more cell cycle progression and metabolism through glycolysis (Ward and Thompson, 2012). However, in vitro over-expression of c-Myc also leads to rapid cell death (Wang et al., 2011); Burkitt's lymphoma cells must have other genetic changes to avoid this consequence. Several reported genetic changes that may serve this function include mutations in p53, Bim, cyclin D3/CDK6, and PI(3) Kinase (Egle et al., 2004; Love et al., 2012). However, none of these is as common as the c-Myc translocation event.

Over-expressing c-Myc alone in mice through introduction of a IgH-c-Myc fusion transgene (EμMyc) indeed can induce B cell lymphoma/leukemia, but the cancer cells do not demonstrate all the features of Burkitt's lymphoma. In fact, many tumors thus derived are pro B or pre B cell lymphoma, and the mature B cell lymphomas do not show characteristics of germinal center B cells (Adams et al., 1985). Other mouse models, such as one that co-overexpress c-Myc and a constitutively active PI (3) kinase in germinal center B cells, demonstrate more similarity to human Burkitt's lymphoma (Sander et al., 2012). Nevertheless, a best mouse model should incorporate mutations or other genetic changes that are commonly found in human tumors and demonstrate human tumor-like behavior, and this requires more knowledge about the genetic derangements in the human Burkitt's lymphoma patients.

Recently, the rapidly developing Next Generation Sequencing (NGS) technology made it possible for investigators to sequence entire genomes of cancer cells and normal cells from multiple patients. Application of this technology to Burkitt's lymphoma led to identification of new common mutations occurring in multiple patients that might contribute to the transformation process of these cells. One of the most commonly mutated genes is the helix-loop-helix transcription regulator, Inhibitor of DNA Binding 3 (Id3). In fact, up to 60% of Burkitt's lymphoma samples carry Id3 mutation, mostly causing loss of Id3 expression or function (Schmitz et al., 2012). Interestingly, similar

studies conducted by our collaborator, Dr. Sandeep Dave's group, showed that Id3 mutation only exists in the tumors with c-Myc mutations and is rarely seen in other B cell malignancies such as diffuse large B cell lymphoma (Love et al., 2012).

As discussed in Chapter 1, deficiency of E proteins and Id proteins has been shown to cause or associate with hematopoietic malignancies in different mouse models and human diseases. Therefore, it is highly likely that Id3 may play a role in the transformation process of Burkitt's lymphoma. In fact, a recent publication pointed out that Id3 and E2A may regulate the activity of B cell receptor signaling, which is important for the survival of Burkitt's lymphoma cells (Schmitz et al., 2012). Dr. Sandeep Dave's group also showed that over-expressing mutant Id3 promoted Burkitt's lymphoma cell proliferation (Love et al., 2012). However, the studies were performed with lymphoma cell lines. It is still unknown at which stage of the transformation process Id3 is mutated and what role it actually plays in vivo.

Paradoxically, we have reported in the past that Id3-deficient B cells actually have impaired proliferation capacity, in contrast to their potential oncogenic role in Burkitt's lymphoma (Pan et al., 1999). This may suggest a unique interaction between Id3 and c-Myc and correlate with the exclusive presence of Id3 mutation in Burkitt's lymphoma. Following the exciting discovery made in Dr. Dave's laboratory, we collaborated with Dr. Dave to investigate genetic interactions between Id3 and c-Myc. As a pilot project, we started the collaboration by establishing Id3-deficient, c-Myc over

expressing mice. We plan to utilize this model to study the role of Id3 in the development of Burkitt's lymphoma-like disease in the mice. The study can help us understand why the mutation of this gene is so common in Burkitt's lymphoma yet so rare in other kinds of B cell malignancies, and whether targeting Id3 and E proteins will be a potential therapeutic option for the treatment of Burkitt's lymphoma.

4.2 Materials and Methods

Mice

The $Id3^{-/-}$ (Pan et al., 1999) and $Id3^{f/f}$ (Guo et al., 2011) mice have been described previously and all maintained on pure B6 background. The EμMyc transgenic mice and AIDCre transgenic mice were purchased from The Jackson Laboratory. Animals were bred and maintained in the SPF facility managed by Duke University Division of Laboratory Animal Research. All animal procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Germinal center induction

To induce germinal center formation, 6 to 8 weeks old mice were immunized with a mixture of 4-Hydroxy-3-nitrophenylacetyl haptenated chicken gamma globulin (NP-CGG) (0.2 mg per mouse) (Biosearch Technologies) and aluminum sulfate (alum)

(100mg per mouse) (EMD Millipore) through i.p. injection. 10 days later, immunized mice were sacrificed, and their splenocytes were harvested for germinal center B cell analysis.

Flow cytometry

The antibodies used in the flow cytometry analyses were as follows: anti-mouse CD4 (GK1.5), anti-mouse CD8a (53-6.7), anti-mouse B220 (RA2-6B2), anti-mouse TCRβ (H57-597), anti-mouse GL7 (GL7), anti-mouse CD38 (90), anti-mouse IgM (RMM-1) and anti-mouse IgD (11-26c.2a) were purchased from Biolegend. The anti-mouse Fas (Jo2) antibody was purchased from BD Biosciences. The anti-mouse Ki67 antibody was purchased from eBioscience. 7-Aminoactinomycin D (7-AAD) was purchased from Life Technologies.

Single-cell suspensions were prepared from tissue samples and suspended in cold FACS buffer (1×PBS supplemented with 5% bovine calf serum). 1×10^6 cells were stained with antibodies in the dark at 4°C for 30 min. After washing with cold FACS buffer, cell suspensions were analyzed on a FACSCanto II flow cytometer (BD Biosciences). FlowJo software (Tree Star) was used for data analysis.

Histology and immunofluorescence

Tissue samples were either fixed in 10% formalin or frozen in OCT compound for subsequent analysis. For routine histology examination, formalin-fixed samples were embedded in paraffin and sectioned, followed by hematoxylin and eosin staining. For immunofluorescence studies, 5 μm frozen sections were made and fixed in 50:50 acetone/methanol at -20℃ for 10 minutes. The sections were then stained with primary antibody at 4℃ overnight, followed by secondary antibody staining and Hoechst 33342 nuclear counter stain (Life Technologies). The slides were mounted in Fluoromount G (SouthernBiotech) and examined with a confocal microscope (SP5, Leica).

Real-time PCR

Germinal center B cells ($B220^{+}GL7^{+}Fas^{+}$) and non-germinal center B cells ($B220^{+}GL7^{-}Fas^{-}$) were sorted from the spleens of NP-CGG/alum immunized mice, and their RNA was extracted with RNaqueous micro kit (Life Technologies). The RNA was reverse transcribed into cDNA with random hexamers and M-MLV reverse transcriptase (Life Technologies). Real-time PCR was performed with a Mastercycler ep realplex (Eppendorf). 18s rRNA was used as an internal control. The primer sequences are: Id3-F, AGCTTTTGCCACTGACCC; Id3-R, AGATCGAAGCTCATCCATGC; Id3-probe, /56-

FAM/TCCCAGAGT/ZEN/CCCAGAGTCCCAG/3IABkFQ/; 18s rRNAF, GTT CCT TTG GTC GCT CGC TCC TC; 18S rRNAR, GGC ACG GCG ACT ACC ATC GA.

Statistical analysis

The real-time PCR data was compared using Student's t test, and p value less than 0.05 was considered significant. The survival lengths of animals with different genotypes were analyzed with Kaplan–Meier survival curves, and different groups were compared using the log rank test. p value less than 0.05 was considered significant.

4.3 Results

4.3.1 Id3 is expressed in both germinal center and non-germinal center B cells

I first sought to determine whether Id3 is expressed in B cells during an immune response by immunizing both wild type and EμMyc transgenic mice with NP-CGG/alum i.p. 10 days after the immunization. Similar percentages of $GL7^+Fas^+$ cells could be detected among the splenic $B220^+$ population in both wild type and EμMyc transgenic mice (**Fig 18A**). I sorted the $B220^+GL7^+Fas^+$ and $B220^+GL7^-Fas^-$ cells and performed real-time PCR to detect Id3 mRNA expression, and I found that Id3 is detectable in both populations from either wild type or EμMyc transgenic mice (**Fig 18B**). Overall, the expression level is very similar across all sample groups; the only significant difference is between the non-germinal center B cells from wild type versus EμMyc

transgenic mice, with the expression being lower in those from the transgenic mice. This is different from the studies performed with Burkitt's lymphoma cell lines; in those experiments, Myc appeared to be an inducer of Id3 expression (Seitz et al., 2011). My finding demonstrated that malignant cells may behave differently from premalignant cells, and in order to truly understand the role of a genetic change in the transformation of a cell population, it is very important to develop a mouse model and observe the impact of the genetic change early in the disease developmental process.

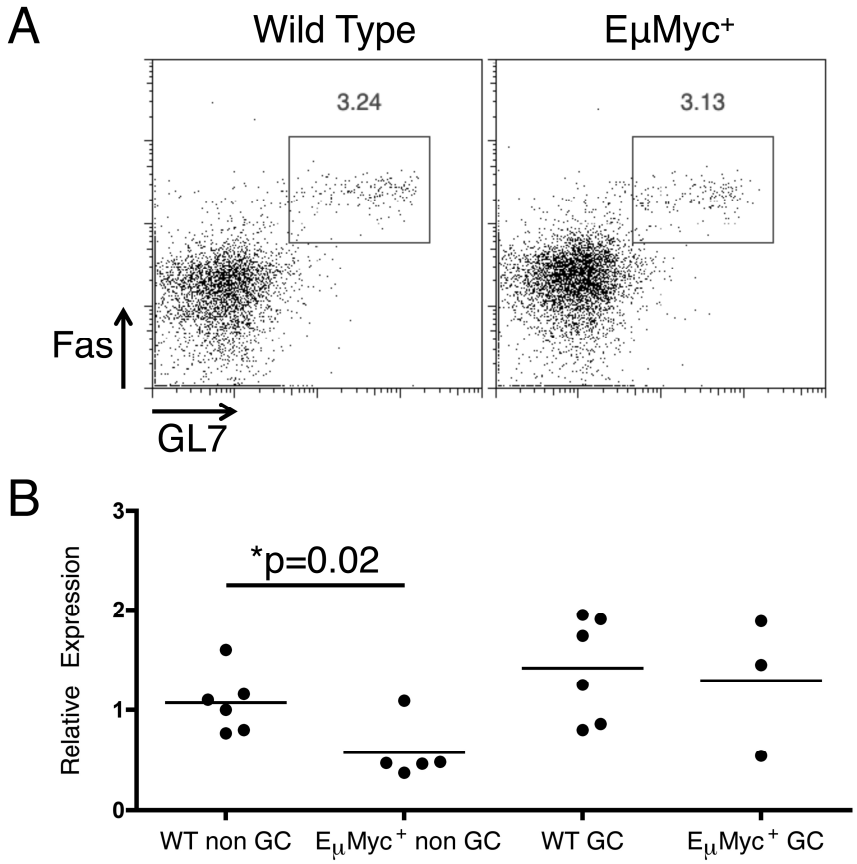


Figure 18: Expression of Id3 among wild type (WT) and EμMyc$^+$ germinal center (GC) and non-germinal center (non GC) B cells 10 days after immunization with NP-CGG.

(A) 10 days after immunization, the percentage of GL7$^+$Fas$^+$ germinal center cells among splenic B220$^+$ cells was similar between wild type and EμMyc$^+$ mice. Data representative of 3 mice in each group. **(B)** The Id3 mRNA expression level across different groups of B cells sorted from the spleen of immunized animals was determined by QPCR. GC: GL7$^+$Fas$^+$; non GC: GL7$^-$Fas$^-$. Each dot represents one mouse.

4.3.2 $Id3^{+/-}$ $E\mu Myc^{+}$ mice succumb to lymphoid tumor faster than $Id3^{+/+}$ $E\mu Myc^{+}$ mice, but the tumors have a T cell origin

After confirming that Id3 is expressed in both germinal and non-germinal center B cells in the mouse, I next sought to determine the effect of Id3 deficiency on the mouse life span and tumor formation in the context of c-Myc over-expression. When comparing the life span between $Id3^{+/-}$ $E\mu Myc^{+}$ and $Id3^{+/+}$ $E\mu Myc^{+}$ mice, I found that $Id3^{+/-}$ $E\mu Myc^{+}$ mice have a reduced survival time (**Fig 19**) (median survival: 70.5 days for $Id3^{+/-}$ $E\mu Myc^{+}$ mice vs. 114.0 days for $Id3^{+/+}$ $E\mu Myc^{+}$ mice, log rank test *p=0.01). The $Id3^{+/-}$ $E\mu Myc^{+}$ mice all die of a lymphoproliferative disease that infiltrates multiple organs including the lymph nodes, spleen, liver, kidney and lung. This result indicates that Id3 is a very important tumor suppressor in the lymphoid system in the context of c-Myc over-expression, and loss of only one Id3 allele can greatly accelerate the oncogenesis process. $Id3^{+/-}$ $E\mu Myc^{+}$ mice rarely survived until reproductive age to enable the production of $Id3^{-/-}$ $E\mu Myc^{+}$ mice.

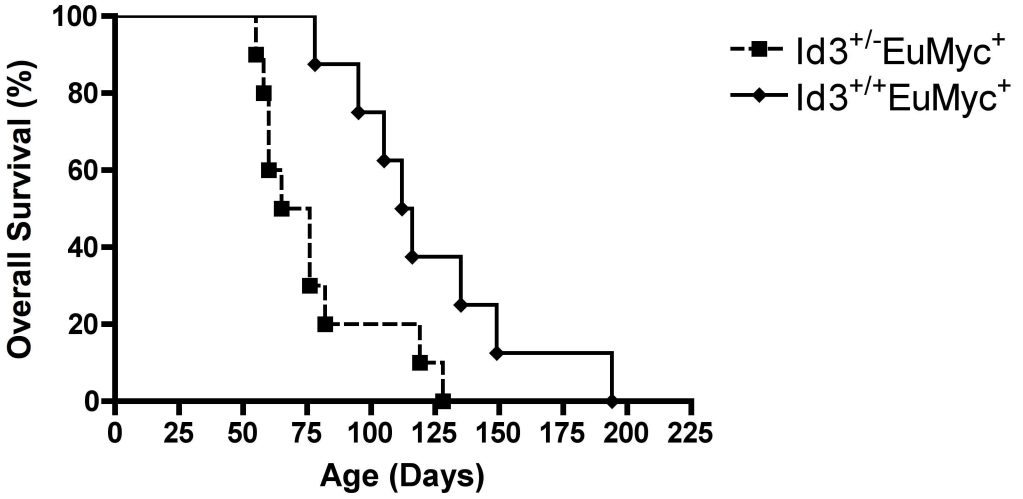


Figure 19: Loss of one copy of Id3 gene reduced survival time of EμMyc transgenic mice

N=10 for $Id3^{+/-}$ $EμMyc^{+}$, n=8 for $Id3^{+/+}$ $EμMyc^{+}$; log rank test *p=0.01.

However, when I analyzed the phenotype of the cancer cells recovered from the $Id3^{+/-}$ $E\mu Myc^{+}$ mice, I found that these cells were not B cells, but they are $TCR\beta^{+}$ T cells (**Fig 20**). Most cells were $CD4^{+}$ $CD8\alpha^{+}$, resembling the double positive (DP) thymocytes. In comparison, the cancer cells from $Id3^{+/+}$ $E\mu Myc^{+}$ mice were B cells (**Fig 20**), as reported previously (Adams et al., 1985). It is known that the $E\mu Myc^{+}$ transgene can be expressed in T cells, and certain mouse strains preferentially develop T cell disease when the $E\mu Myc^{+}$ transgene is introduced (Yukawa et al., 1989). My result indicates that c-Myc over-expressing T cells or T cell progenitors are more prone to cancer development than B cells in the C57BL6 genetic background, but Id3 was very efficient in suppressing this process, so in $Id3^{+/+}$ animals the B cell malignancy can develop at a later time point. How Id3 suppresses c-Myc-driven oncogenesis in T cells, and whether the mechanism is similar or different from that in B cells, will be one objective of our future studies.

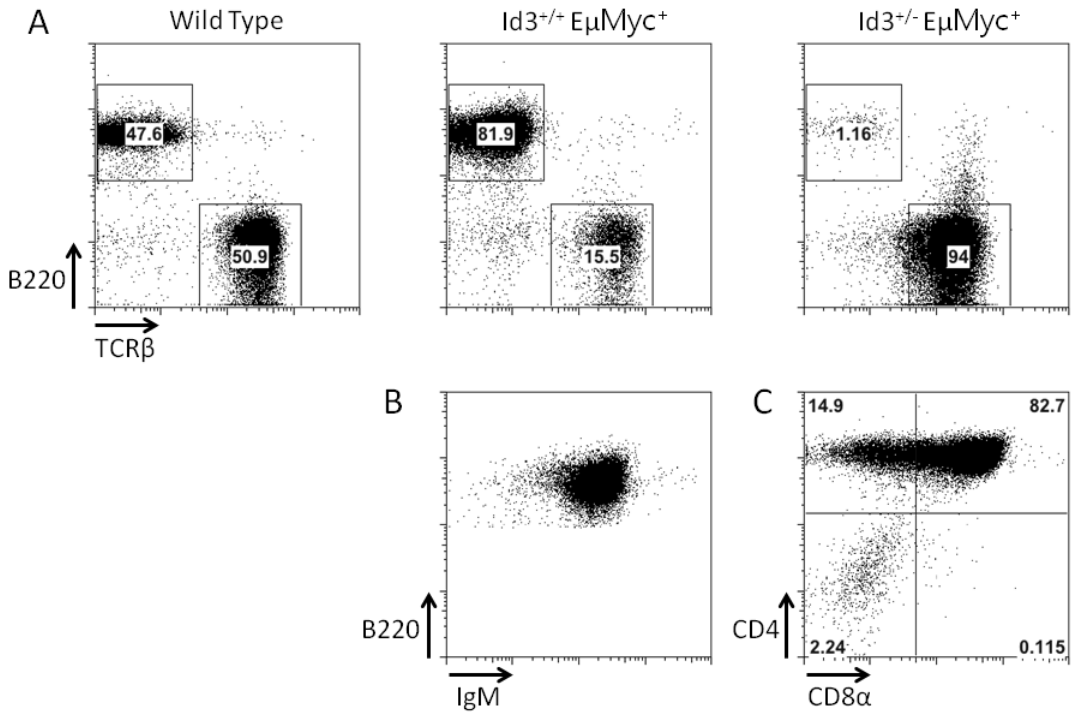


Figure 20: Id3$^{+/-}$ EμMyc^{+} mice develop T cell lymphoma

(A) Comparing cells from a normal lymph node of a wild type mouse to cells from tumors of Id3$^{+/+}$ EμMyc^{+} mice and Id3$^{+/-}$ EμMyc^{+} mice. While Id3$^{+/+}$ EμMyc^{+} tumor cells are mostly B220^{+} B cells, Id3$^{+/-}$ EμMyc^{+} tumor cells are TCRβ^{+} T cells. **(B)** The B220+ cells from Id3$^{+/+}$ EμMyc^{+} mice are also IgM+. (C) The TCRβ^{+} T cells from Id3$^{+/-}$ EμMyc^{+} mice are mostly CD4^{+}CD8α^{+}. Data representative of three mice in each group.

4.3.3 Germinal center B cell-specific Id3 knockout, EμMyc$^+$ mice may have accelerated B cell lymphoma formation with some Burkitt's-like features

The above study demonstrated the tumor suppressor role of Id3 in the context of c-Myc over-expression in T cells. However, the rapid generation of T cell lymphoma prevents further analysis of any possible role of Id3 in the development of Burkitt's lymphoma. Therefore, I switched to a system with more limited and specific deletion of Id3, utilizing the Id3$^{f/f}$ AIDCre$^+$ mice. AID (activation-induced cytidine deaminase) is an enzyme that is specifically highly expressed in germinal center B cells, the supposed origin of human Burkitt's lymphoma. AIDCre can drive efficient deletion of floxed genes in the germinal center B cells (Crouch et al., 2007). Preliminary results showed that the Id3$^{f/f}$ AIDCre$^+$ EμMyc$^+$ mice developed B cell, not T cell, lymphoma (**Fig 21A**). These tumors do show some features similar to human Burkitt's lymphoma, such as a histology feature called "starry sky", indicating rapid proliferation of tumor cells and spontaneous apoptosis (**Fig 21B**). Immunofluorescence staining showed that nearly 100% of cells express Ki-67, a marker for cells actively going through cell cycles (**Fig 21C**). Flow cytometry also showed that they express some markers comparable to germinal center B cells (**Fig 21A**). Whether the Id3$^{f/f}$ AIDCre$^+$ EμMyc$^+$ mice have a shorter life span than the Id3$^{f/f}$ AIDCre$^-$ EμMyc$^+$ mice cannot be concluded until we accumulate more survival data.

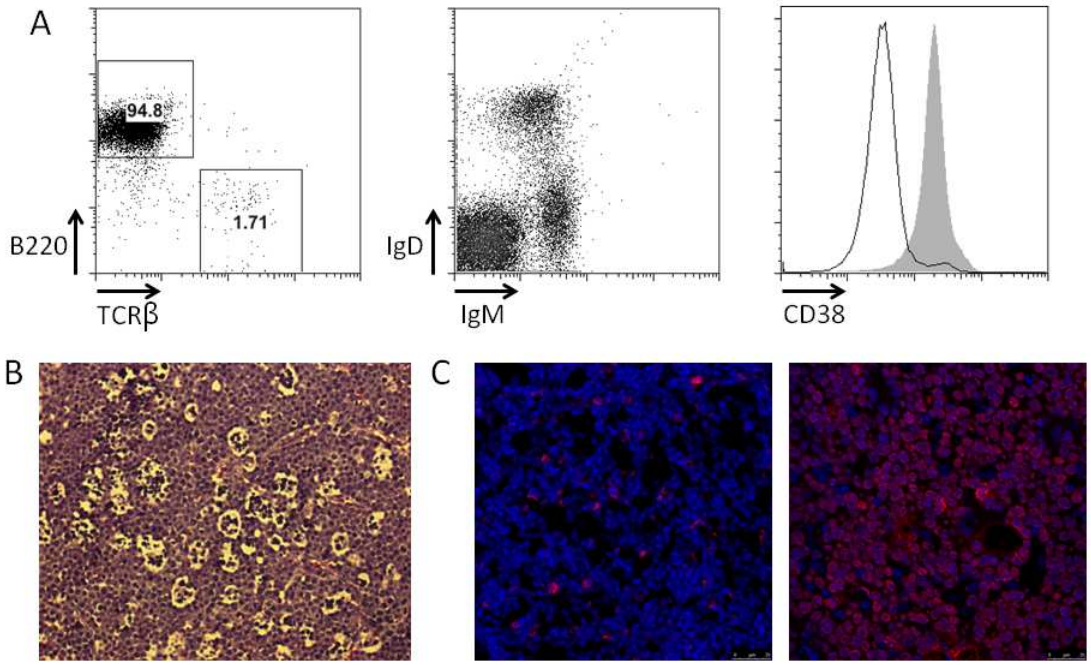


Figure 21: Tumor cells from Id3$^{f/f}$ AIDCre^{+} EμMyc^{+} mice show features of germinal center B cells

(A) The tumor cells from Id3$^{f/f}$ AIDCre^{+} EμMyc^{+} mice are mostly B220^{+} B cells. A large proportion of these cells is IgM^{-}IgD^{-}, and they express lower levels of CD38 (line histogram) comparing to B cells from a wild type mouse spleen (gray shaded histogram), characteristic of germinal center B cells. **(B)** Hematoxylin & eosin staining of the tumor tissue showed a "starry sky" pattern. **(C)** Immunofluorescence staining showed that the tumor tissue (right) is strongly positive of Ki67, as compared to the low level staining of normal lymph node tissue from a wild type mouse (left). Red: anti-Ki67. Blue: Hoechst 33342.

4.4 Discussion

Our study demonstrated that Id3 indeed can function as a tumor suppressor in the lymphoid system in the context of c-Myc over-expression, at least in the T cell lineage. When Id3 is specifically deleted in the germinal center B cells, EμMyc transgenic mice can indeed develop a Burkitt's-like B cell disease. This result is compatible with the previous human Burkitt's lymphoma sequencing studies and the Burkitt's lymphoma cell line studies.

In order to further confirm that Id3 deficiency potentiate the development of Burkitt's-like disease in the mouse, we need to collect more animals to establish a survival difference between $Id3^{f/f}$ $AIDCre^{+}$ $E\mu Myc^{+}$ mice and $Id3^{f/f}$ $AIDCre^{-}$ $E\mu Myc^{+}$ mice. We need to examine more features of germinal center B cells, such as Bcl6 expression (Basso and Dalla-Favera, 2012), to determine whether the cancer cells truly have a germinal center origin, as Bcl6 is considered the master transcriptional factor of germinal center B cells as well as a hallmark of Burkitt's lymphoma. So far, limited analysis with other classical mouse germinal center markers, such as GL7 and Fas, revealed that some tumor cells show a general increase of both markers but below the levels seen in wild type germinal centers, while other tumors contain a fraction of cells expressing only Fas or GL7 (data not shown). It is known that tumor cells sometimes do not retain all of the surface markers of their cells of origin (Longo, 2011); a standardized panel of germinal

center specific/related markers will be required to best describe the identity of these tumor cells arising in the Id3 conditional knockout, EμMyc^{+} mice.

In addition to surface markers and transcription factors, two other important pieces of evidence of germinal center history will be immunoglobulin isotype switching and somatic hypermutation. We plan to perform more flow cytometry analysis of tumor cells to detect any product of isotype class switching, such as surface IgG or IgA expression. We will also sequence the immunoglobulin genes of the tumor cells, comparing them to germline sequences to detect evidence of somatic hypermutation.

Although analysis of wild type and EμMyc transgenic mice did not show significant difference in Id3 expression between germinal center B cells and non-germinal center B cells, Id3 expression is still detected in the germinal center, and specific deletion of Id3 in germinal center B cells may perturb normal GC responses. We will try to induce germinal center responses in Id3$^{f/f}$ AIDCre^{+} EμMyc^{+} mice and Id3$^{f/f}$ AIDCre^{-} EμMyc^{+} mice to determine if the absence of Id3 results in any advantage for the germinal center population prior to malignant transformation. These results will help us clarify how Id3 cooperates with c-Myc in transforming B lymphocytes.

In order to compare our model with human Burkitt's lymphoma and determine how similar this model is to human disease, we will perform whole exome sequencing and RNA sequencing to obtain the mutation and gene expression profile of the tumor cells from Id3$^{f/f}$ AIDCre^{+} EμMyc^{+} mice. These methods have been used previously to

validate another mouse model of Burkitt's lymphoma (Sander et al., 2012). If our model can be shown to closely resemble human Burkitt's lymphoma, it can potentially be a better model since it captures two of the most common genetic drivers of the disease: Myc over-expression and Id3 loss. The model may be useful for further dissecting the oncogenesis mechanisms as well as testing experimental therapies. Comparing this model with other tumor models associated with loss or gain of E proteins/Id proteins may also inform us how the same genes can switch between the role of oncogenes and tumor suppressor genes in different tissue and developmental stages.

5. General discussions and future directions

Through these three projects we have once again demonstrated the versatility of the E – Id gene regulation network. The same Id2 protein can inhibit the survival of γδT cells during development but sustain the survival of CD4 T cells during an immune response. The same E2A protein can inhibit T cell lymphoma development in the wild type mice but may be promoting T cell lymphoma development in the context of c-Myc over-expression. These apparently contradictory functions of the same genes naturally lead us to ask the next question: how can this be achieved at the molecular level?

There are several possible mechanisms. First, E proteins may be regulating different sets of genes and pathways in different cells at different developmental stages (Lin et al., 2012a). Epigenomic modifications and chromatin accessibility differences may dictate which E-box containing gene is susceptible to E protein regulation in a particular cell type. Second, some genes may be regulated by E proteins in a similar manner across cell types, but different cell types have different reliance on each gene, so the net outcome becomes different. For example, E proteins may up-regulate one anti-apoptotic gene (such as Bcl-2 (Cannarile et al., 2006)) but also up-regulate another pro-apoptotic gene (such as Bim (Schwartz et al., 2006)); the cells that depend on the first gene will experience a survival advantage when E protein activity is high, but the cells that are sensitive to the second will experience increased apoptosis. Third, although there are no

such examples reported yet, theoretically E proteins may bind to the same target genes across several cell types but up-regulate the genes in some cells while down-regulate them in others, through recruiting different transcription co-activators or co-repressors. Although the E protein binding to the target DNA sequence may be the same, the co-repressor (such as ETO (Zhang et al., 2004)) or co-activator (such as p300 (Qiu et al., 1998)) available in the cell may be different, thus resulting in opposite effects on the expression of the target genes.

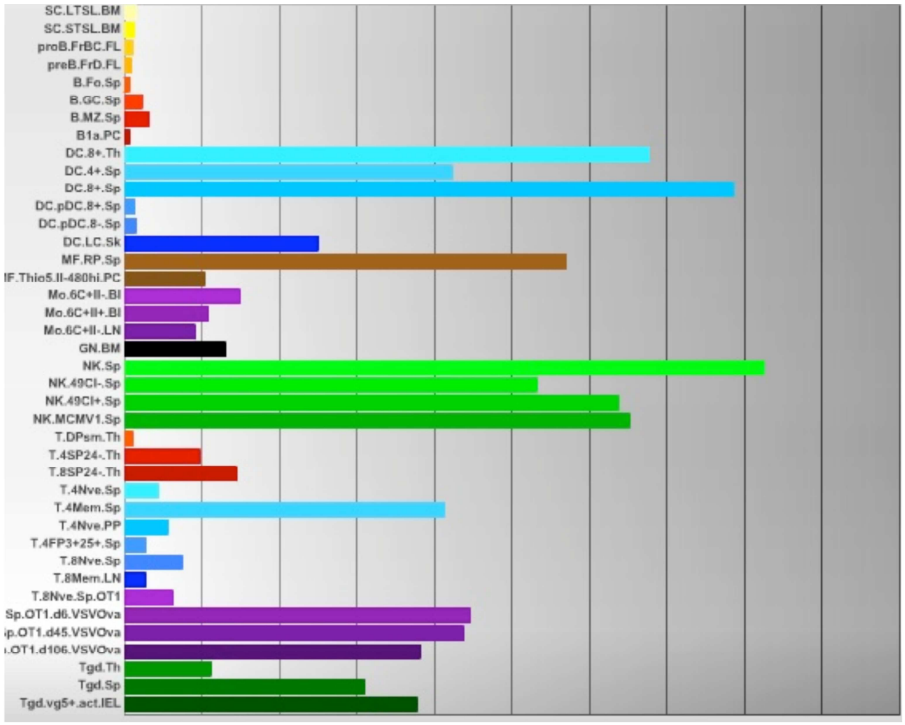
To differentiate among these three possibilities, we may turn to the new high throughput chromatin immunoprecipitation-sequencing (ChIP-Seq) method to examine the activity of E proteins in different situations (Lin et al., 2012a). By recording the position of E proteins in the entire genome, we will understand whether it is regulating different genes and pathways in different cell types. Combining the ChIP-Seq data with gene expression profiling methods such as microarray or RNA-Seq, we may deduct whether E proteins are up-regulating or down-regulating one particular set of genes, and whether its effect is the same or different in different cell types. This research method will be particularly powerful in the study of phenomena that appears to be "fine tuned" by E protein activity levels, such as the development of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells discussed in Chapter 2, or the selective expansion of NKT cells versus $V\gamma1.1^{+}V\delta6.3^{+}$ T cells in $Id2^{f/f}$ $Id3^{f/f}$ $LckCre^{+}$ mice with wild type or partially deleted E protein genes (Li et al., 2013). In these cases, the molecular difference is subtle; E protein activity may be

moderately higher in one situation than in another, yet the result can be dramatically different. Do E proteins bind to loci normally not easily accessible in the cell only when there is an E protein excess? Do they influence the expression of some genes to a greater extent than others when the E protein activity is higher? Can we predict such difference by analyzing the sequences adjacent to the E-box binding site, also inferring potential partners of E proteins in the process? Analyzing the genome-wide localization of E proteins and the expression of genes bound by E proteins can help us find clues to answer these questions.

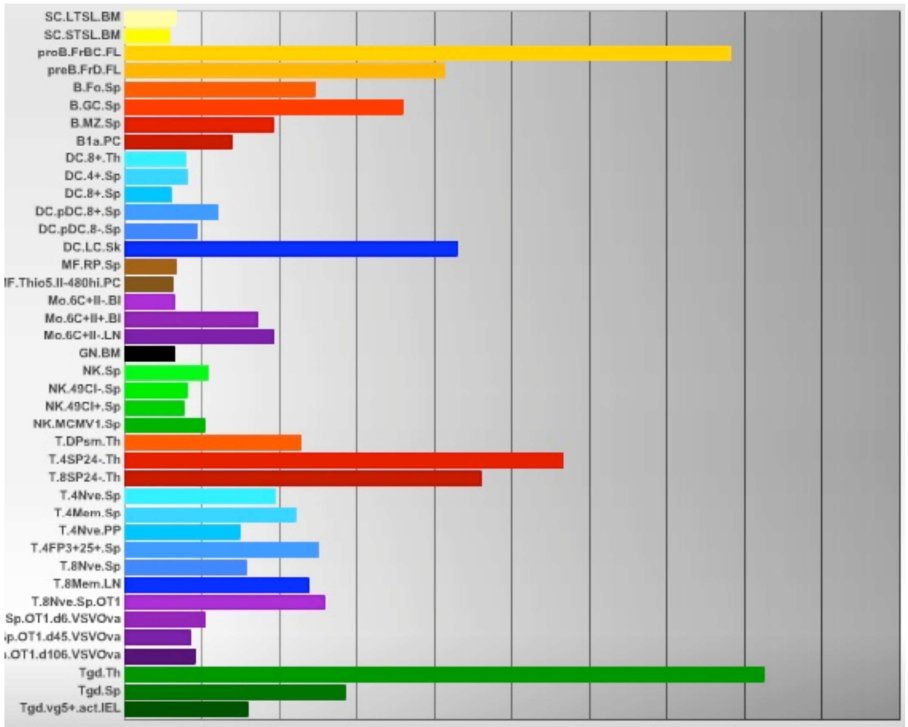
On the side of the Id proteins, the situation initially appears to be simple. No matter whether the cell expresses Id2 or Id3, the result may be the same: E protein activity will be inhibited. However, evolution nevertheless created two Id proteins to be expressed in the mammalian immune system, and two other homologs (Id1, Id4) exist and are expressed in other tissues. In my study, I described many different functions that are carried out by Id2 or Id3, respectively: (1) Id3 plays a dominant role in limiting the expansion of $V\gamma1.1^{+}V\delta6.3^{+}$ cells, while Id2 plays a moderate role in limiting maturation of γδ T cells in general; (2) Id2 sustains the effector cell population of CD4 T cells, and its role cannot be compensated by Id3. In previous reports, Id2 and Id3 have also been shown to control different populations of memory CD8 T cells (Yang et al., 2011); (3) while both Id2 and Id3 are expressed in the germinal center B cells, with Id2 being highly expressed (Schmitz et al., 2012), only Id3 mutation was detected in the

germinal center B cell-derived Burkitt's lymphoma. As previously described, all the innate lymphoid cells, including natural killer cells (NK), lymphoid tissue inducer cells, and a variety of cytokine producing, helper T cell-like innate lymphoid cells, depend on Id2 for their development, not Id3(Mjosberg et al., 2012). It was only in the development of SP thymocytes from DP thymocytes, and in the development of NKT cells, that a mutually compensating relationship between Id2 and Id3 has been clearly demonstrated, since only double conditional knockouts of both genes show a significant phenotype (Jones-Mason et al., 2012; Li et al., 2013). How and why does the organism use two or more Id proteins to regulate E proteins to achieve these different effects?

Gene expression analysis showed that the mRNA level of Id2 and Id3 are different in many cell types of the immune system (**Fig 22**). This different expression pattern may directly explain part of the functional difference between the two genes. For example, Id2 is highly expressed in the NK cells while Id3 is not, thus explaining the phenomenon that NK cells require Id2 but not Id3 for their development. The *cis*-regulatory elements in the DNA sequence that determine this differential expression pattern remains to be discovered.


SC.LTSL.BM
SC.STSL.BM
proB.FrBC.FL
preB.FrD.FL
B.Fo.Sp
B.GC.Sp
B.MZ.Sp
B1a.PC
DC.8+.Th
DC.4+.Sp
DC.8+.Sp
DC.pDC.8+.Sp
DC.pDC.8-.Sp
DC.LC.Sk
MF.RP.Sp
IF.Thio5.II-480hi.PC
Mo.6C+II-.Bl
Mo.6C+II+.Bl
Mo.6C+II-.LN
GN.BM
NK.Sp
NK.49CI-.Sp
NK.49CI+.Sp
NK.MCMV1.Sp
T.DPsm.Th
T.4SP24-.Th
T.8SP24-.Th
T.4Nve.Sp
T.4Mem.Sp
T.4Nve.PP
T.4FP3+25+.Sp
T.8Nve.Sp
T.8Mem.LN
T.8Nve.Sp.OT1
Sp.OT1.d6.VSVOva
ip.OT1.d45.VSVOva
.OT1.d106.VSVOva
Tgd.Th
Tgd.Sp
Tgd.vg5+.act.IEL

Id2


SC.LTSL.BM
SC.STSL.BM
proB.FrBC.FL
preB.FrD.FL
B.Fo.Sp
B.GC.Sp
B.MZ.Sp
B1a.PC
DC.8+.Th
DC.4+.Sp
DC.8+.Sp
DC.pDC.8+.Sp
DC.pDC.8-.Sp
DC.LC.Sk
MF.RP.Sp
IF.Thio5.II-480hi.PC
Mo.6C+II-.Bl
Mo.6C+II+.Bl
Mo.6C+II-.LN
GN.BM
NK.Sp
NK.49CI-.Sp
NK.49CI+.Sp
NK.MCMV1.Sp
T.DPsm.Th
T.4SP24-.Th
T.8SP24-.Th
T.4Nve.Sp
T.4Mem.Sp
T.4Nve.PP
T.4FP3+25+.Sp
T.8Nve.Sp
T.8Mem.LN
T.8Nve.Sp.OT1
Sp.OT1.d6.VSVOva
ip.OT1.d45.VSVOva
.OT1.d106.VSVOva
Tgd.Th
Tgd.Sp
Tgd.vg5+.act.IEL

Id3

Figure 22: Relative expression of Id2 and Id3 mRNA in major cell types of the immune system

Data obtained from the Immunological Genome Project website (https://www.immgen.org/)

In addition to difference in mRNA expression levels, the 3'untranslated regions (UTRs) of the Id2 and Id3 mRNAs also show significant differences (**Fig 23**), suggesting possible differences in post-transcriptional regulations. Analysis of potential microRNA recognition site on the 3'UTRs showed no conservation between the two genes, indicating that they may respond very differently to RNA interference control. Some of my *in vitro* preliminary experiments showed that the 3'UTR of Id3 indeed has the ability to reduce luciferase protein expression when attached to the luciferase mRNA (data not shown). The effect appeared to be mediated by microRNA regulation; therefore, this is very unlikely to be shared between Id2 and Id3. This shows yet another layer of regulation different cell types may utilize to control the two Id genes separately.

Finally, at the protein level, the two Ids also have some important differences (**Fig 24**). Their dimerization interface sequences in the helix-loop-helix domain, although highly similar, still show some amino acid sequence discrepancies, suggesting that they may have different binding affinity with different E proteins. Id2 also contains one nuclear export signal domain that is not present in Id3, which may influence its intracellular localization and its access to the target E proteins.

```
Id2             ATAAATGGCATTTGGGGACTTTTTTTTTTCTTTTTACTTTCTCTTTTTCTTTTGCACAAG 60
Id3             ----------CCCGG----------TCGTCCTGGCACCTCCC----------------G 23
                          **          *  ** *   ** * *                 *

Id2             AAGAAGTCTACAAGATCTTTTAAGACTTTTGTTATCAGCCATTTCACCAGGAGAACACGT 120
Id3             AA------CGCAGG---------------TGCTGGCGCCCGTTCCGCTTGGG--ACCCTG 60
                **        ** *               ** *  *  ** ** * *  **   ** *

Id2             TGAAT---GGACCTTTTTAAAAA--GAAAGCGGAAGGAAAACTAAGGATGA---TCG-TC 171
Id3             GGACTCTGGGACCCTCTCTCCAGCCGGAAGCCTGAGGG----CATGGATGAGCTTCGATC 116
                 ** *   ***** * *    *   * ****   ***      * ******   *** **

Id2             TTG-CCCAGGTGTCGTTCTCCGGCCTGGACTGTGATACCGTTATTTATGAGAGACTTTCA 230
Id3             TTAACCCAGCCCTCTTCACTTACCCTGAACT-CAACGCC----TCGAGGCTGGACCT--G 169
                **  *****   ** *       **** ***   *  **    *  * *   *** *

Id2             GTGCCCTTTCTACAGTTGGAAGGTTTTCTTTATATACTATTCCCACCATGGGGAGCGAAA 290
Id3             GAGCCC--------GAGAGAAGG-----------ACTG-----AACTTGGGTGGC---- 200
                * ****        *   *****            ***      * * ****  **

Id2             ACGTTAAAAAAAAAAGAAAAAAATCACAAGGAATTGCCCAATGTAAGCAGACTTTGCCTT 350
Id3             ----CTGAAGAGCTAGCACACGCTGGTCAGCAGCTGGGCAACGTCA-----CTCTGTCCC 251
                       ** *   ** * *   *    ** *  **  *** ** *     ** ** *

Id2             TTCACAAAGGTGGAGCGTGAATACCAGAAGGACCCAGTATTCGGTTACTTAAATGAAGTC 410
Id3             CACCCTGA-------CTCAAGT--CTAAAAGACTGGCTTTTCCGA-----GAATGGGGT- 296
                  * *  *       *   * *  *  ** ***    * *** *       ****  **

Id2             TTCGGTCAGAAATGGCCTTTTTGACACGAGCCTACTGAATGCTGTGTATATATTTATATA 470
Id3             ----GTC-GAGAGGG----TGTGGGGGGATGCGAGTGGCTGCCCTGCGCAC--------- 338
                    *** ** * **    * **    **  * * **  ***  **   *

Id2             TAAATATATATATATTGAGTGAACCTTGTGGACTCTTTAATTAGAGTTTTCTTGTATAGT 530
Id3             ----------TCTGCCAAG-GCAGCATAAGAGCTGTTC--TTCTGGTTTCCTTG------ 379
                          * *    ** * * * *  *  ** **   **   **** ****

Id2             GGCAGAAATAACCTATTTCTGCATTAAAATGTAATGACGTACT-TATGCTAAACTTTTTA 589
Id3             ----GAGAAAAGCT-CTGCTGCCCTGA----TTATGA---ACTCTATAATAGAGTATATA 427
                    ** * ** **  * ****  * *    * ****   *** ***  ** * * * **

Id2             TAAAAGTTTAGTTGTAAACTTAACCCTTTTATACAAAATAAATCAAGTGTGTTTATTGAA 649
Id3             -----GCTT--TTGTA--------CCTTTTTTACAGGA------AGGTGACTTT-CTG-- 463
                     * **  *****        ****** ****  *      * ***  ***  **

Id2             TGTTGATTGCTTGCTTTATTTCAGACAACCAGTGCTTTGATTTTTTTTATGCTATGTTAT 709
Id3             ---TAATCATGTGATGTATATTAAAC-----------------TTTTTAT---------- 493
                   * **    ** * *** * * **                 *******

Id2             AACTGAACCCAAATAAATACCAGTTCAAATTTATGTAGACTGTATTAAGATTATAATAAA 769
Id3             ----------AAA--------AGTTAACATTT--------TGCAT---------AATAAA 518
                          ***        **** * ****        ** **         ******

Id2             ATGTGTCTGA-CATCAAAAAAAAAAAAAAAAAAA 802
Id3             CCATTTTTGAACACTTTGAAAAAAAAAAA----- 547
                   * * *** **     ***********
```

Figure 23: Alignment of Id2 and Id3 3'UTR sequences

Star (*) indicates identical sequence; colon (:) and period (.) indicate mismatches; dash (-) indicates insertion or deletion. Underlined sequence indicates predicted microRNA binding regions. Notice that no predicted microRNA binding site is shared between Id2 and Id3. Alignment generated by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). MicroRNA binding predicted by microRNA.org (http://www.microrna.org/).

```
Id2             MKAFSPVRSVRKN--SLSDHSLGISRS--KTP-VDDPMSLLYNMNDCYSKLKELVPSIPQ 55
Id3             MKALSPVRGCYEAVCCLSERSLAIARGRGKSPSTEEPLSLLDDMNHCYSRLRELVPGVPR 60
                ***:****.  :   .**::**.*:*.  *:* .:::*:** :**.***:*:****.:*:

Id2             NKKVTKMEILQHVIDYILDLQIALDSHPTIVSLHHQRPGQNQASRTPLTTLNTDISILSL 115
Id3             GTQLSQVEILQRVIDYILDLQVVLAEP---------APGPPDGPHLPIQT---------- 101
                ..::::::****:*********:.* .           **  :..: *: *

Id2             QASEFPSELMSNDSKVLCG 134
Id3             -AELTPELVISKDKRSFCH 119
                 *.  *. ::*:*.: :*
```

Figure 24: Alignment of Id2 and Id3 protein sequences

Star (*) indicates identical sequence; colon (:) and period (.) indicate mismatch but chemically similar residues; dash (-) indicates insertion or deletion. The underlined Id2 amino acid sequence 38-79 forms the dimerization interface in its helix-loop-helix domain, which is very similar but not identical to the corresponding sequence in Id3. Id2 amino acid sequence 106-115 is an identified nuclear export signal, which is absent in Id3. Alignment generated by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Domain information obtained from NCBI database (http://www.ncbi.nlm.nih.gov/).

In summary, Id2 and Id3 have diverged in every aspect from gene expression to protein sequence and appear to have evolved to play different functions for a long time. Although in the beginning, these Id genes may have arisen through gene duplication to serve the kind of "dual safety" mechanisms as described in chapter 2 of this thesis, as time went on, they progressively picked up more and more mutations or variations and became more versatile to execute functions unique to each gene. This phenomenon reminds us that whenever we study genes within a multi-member family, we should always survey both the similarity and diversity of the family members.

Through our studies, we attempt to understand the role of E proteins and Id proteins in order to better understand how the immune system works, in the hope of generating useful knowledge to improve human health. Our attempt to build a mouse Burkitt's lymphoma model based on observed human genetic changes is clearly a direct effort in this direction. The genetically modified mouse models built in this way have advantages compared with those traditional mouse models of human diseases induced by chemical treatment or those arising spontaneously without specified genetic changes. Because the models we study share characteristic genetic changes with human patients, the pathobiology we learn from them may be more applicable to human. However, one must still be cautious because even though we engineer one or two key genetic changes into the mouse and can produce a similar disease phenotype, true human diseases are

still more heterogeneous, and other minor genes might be involved in the pathogenesis process. Nevertheless, as the knowledge of the genetic landscape of different human diseases increase, and our capability of simultaneously manipulating multiple genes in the animal models increases, this approach will lead to more and more realistic animal models in the future.

However, oftentimes original observations made in the mouse cannot be translated to human. For example, although our lab found that Id3 knockout mice spontaneously develop Sjogren syndrome (Li et al., 2004), Id3 mutation has not been identified in human Sjogren syndrome patients (Sellam et al., 2008), leading to some doubt about the relevance of this mouse model to human diseases. I believe we should not be discouraged from studying the Id3 knockout Sjogren syndrome model or other mouse models just because of these negative reports. First, despite all the discrepancies, human biology and mouse biology still share a great amount of similarities; the mouse model remains the best available experiment model for scientists to ask a large number of questions within a reasonable budget. Second, I believe more successful translation of mouse research requires a much more precise comparison between the two species, targeting the right patient population and the right cell population. In our Burkitt's lymphoma mouse study, we found that deleting Id3 in all mouse cells combined with c-Myc over-expression did not lead to B cell lymphomas but led to T cell lymphomas. When we limited the deletion to germinal center B cells, we observed tumors of B cell

origin that is more similar to human Burkitt's lymphomas. The same selectivity should be essential when trying to translate the research in the opposite direction. It is possible that Id3 may be involved in the pathogenesis of a subset of human Sjogren syndrome. This disease is impressively diverse and has several primary and secondary forms, and failing to detect Id3 mutation in a general patient population does not rule out its role in any subsets. Moreover, Id3 mutation, or down-regulation, most likely occurs only in the effector immune cells infiltrating the patients gland tissues, since germline deletion of Id3 may have multiple effects, and humans with complete loss of Id3 may not survive. Like many mutations or other genetic changes occurring in tumor cells, these changes are most likely somatic, so sequencing the germline DNA is unlikely to detect them. Fortunately, with the progress of modern sequencing technology, we are now increasingly capable of accurately categorizing patients with apparently similar diseases into mechanistically distinct groups, using smaller and smaller amount of tissue samples. Comparing the phenotype between animal models and human patient groups may allow us eventually to identify the correct relationship between the two.

Even if there is genuinely no direct counterparts between human and mouse in terms of certain gene-disease relationship, very likely they may still share common downstream disease evolution mechanisms. For example, various mouse models of lupus-like diseases are induced by different genetic modifications, but they all share the production of autoantibodies and several lupus-like end organ damages, such as

glomerulonephritis. Even though Id3 mutations have not yet been identified in human Sjogren syndrome patients, they do share the features of exocrine gland infiltration and fibrosis with the Id3 knockout mouse models. The mechanisms involved in these processes, such as the excessive production of IL-13 observed in mice (Mahlios and Zhuang, 2011), may also occur in human, even though it might be triggered by a genetic change other than Id3 mutation. If this is proven to be the case, anti-IL13 therapy may well benefit patients with Sjogren syndrome. The translation of finding the role of IL-17 in psoriasis development in mouse models (Cai et al., 2011) into successful anti-IL-17 treatment for psoriasis patients (Leonardi et al., 2012; Papp et al., 2012) is a great example; although human psoriasis certainly is not induced by the same chemical as in certain mouse model (Cai et al., 2011), therapies targeting the same pathway benefits both. I will always keep this, and all what I have learned through the studies of E proteins and Id proteins, in mind to serve as my permanent motivation for future scientific researches.

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